Phase Change of Bacterial Community by Residual Antibiotic Contamination for Nitrogen Removal in Wastewater Treatment

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Abstract

Nowadays, antibiotics are ubiquitous environmental contaminants discharged from pharmaceutical factories, medical facilities, animal husbandry and domestic sources. One frequently detected class of synthetic antibiotics, quinolones, play an important role in the treatment of serious bacterial infections. Bacteria cannot distinguish such as levofloxacin, ciprofloxacin, norfloxacin and ofloxacin, all of which belong to quinolones, with the same inhibition mechanism for bacterial growth. Thus, in this case, bacteria are exposed to the total quinolone concentrations. In addition, many kinds of quinolone antibiotics could reach wastewater treatment plants (WWTPs) from various sources and they are usually detected at levels ranging from ng/L up to mg/L.

Levofloxacin (LVX), a new quinolone type and widely used quinolone antibiotics, is mostly utilized in Japan. In addition, LVX could not be completely metabolized in humans and animals while maintaining its activity, which also cannot be completely removed during wastewater treatment using the current technologies. Thus, after being utilized, it would be discharged into the environment eventually and transferred to other environmental compartments, spreading the problem to many other related ecosystems, such as multi-resistant bacteria and antibiotic resistance genes (ARGs). Importantly, the reason why most WWTPs look like working well till now even though lots of antibiotic residues might already exist in the wastewater needs to be explored. In addition, up to now, very little information could be found on how microbial community adapts when exposed to the residual antibiotics.

In this work, sequencing batch reactor (SBR) was used as it has been applied widely for small-scale WWTPs due to its incomparable advantages including single-tank configuration, small foot print, easily expandable, simple operation and low capital costs. To mimic the uncontrolled or accidental discharge of LVX to the WWTPs, both exposure and re-exposure of varying LVX concentrations were considered and designed in the study. In addition to the effect on nutrients removal from the wastewater, dynamic changes of microbial communities during both exposure and re-exposure of LVX were also recorded and analyzed. This study aimed to shed light on the acclimation mechanisms of bacteria to acute and chronic/fluctuated LVX levels in SBRs.

The results of minimum inhibitory concentration (MIC, 32 mg-LVX/L) and minimum bactericidal concentration (MBC, 512 mg-LVX/L) of the sampled sludge showed that the LVX resistance/tolerance for bacterial growth has already existed in sludge from the actual WWTPs

(The LVX concentration levels of the directly filtered inoculated sludge samples were in the range of 0.09 to 0.76 mg-LVX/L in this study.). LVX addition could inhibit the uptake of organics by the microorganisms, and a better dissolved organic carbon (DOC) removal performance during re-exposure was noticed in the reactor with lower LVX exposure concentration experience. Still, at least 50% of the influent LVX remained in the effluent. NH_4-N removals were remarkably affected, most probably the inhibition of ammonia-oxidizing bacteria (AOB) by the high concentration of LVX, resulted in much lower nitritation efficiency. Ammonia oxidation ability recovered much slower during re-exposure. Overall, the NO_2-N accumulation as well as lower NO_3-N production were observed during the exposure/re-exposure to the higher concentration of LVX. During Stage V, NO_2-N concentration in both R2 and R3 could not recover as the beginning level and the nitratation processes are still inhibited in R2 and R3. TN increased with the LVX exposure/re-exposure, might not only be attributable to inhibited denitrification, but also the addition of LVX ($C_{18}H_{20}N_3O_4F$) which contains 11.7% of N (w/w). Re-exposure to 128 mg-LVX/L exerts more negative effects on the TN level of R3 than R2. The recovery of biological nutrients removal performance was retarded by LVX exposure, due to the fact that the key bacteria, i.e. *Nitrosomonas* sp., (AOB) and *Nitrospira* sp., nitrite-oxidizing bacteria (NOB) decreased, and that *Thauera* sp., the predominant denitrifiers were reduced during the LVX exposure period. However, after stopping exposure these population was quickly increased and thus the performance was recovered. The results of the non-metric multidimensional scaling (NMDS) and microbial community by sequencing showed the LVX concentration was a crucial factor to phase change of bacterial community controlling the quantitative evolution of the communities in the reactor systems. This effect was more pronounced as the LVX concentration was higher. Proteobacteria phylum always dominated the control reactor, but the proportion shifted dramatically with the addition of LVX. The results from this study suggest the control of residual antibiotics concentration in WWTPs is important to control antibiotic resistant bacteria (ARB) in WWTPs.

The findings from this work are expected to provide good guidance on performance recovery of WWTPs when encountering accidental loading of antibiotics like LVX. Also, the results from this work could shed light on better understanding the acclimation mechanisms of bacteria and bacterial community to fluctuated LVX levels exposure situation during wastewater treatment.

Keywords: Levofloxacin (LVX); Adaptation; Nitrogen and phosphorus removals; Microbial community; Sequencing batch reactors (SBRs)

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Abbreviations

AOB:	Ammonia-oxidizing bacteria
ARB:	Antibiotic resistant bacteria
ARGs:	Antibiotic resistance genes
COD:	Chemical oxygen demand
DDD:	Defined daily doses
DO:	Dissolved oxygen
DOC:	Dissolved organic carbon
HPLC:	High-performance liquid chromatography
HRT:	Hydraulic retention time
HTS:	High-throughput screening
LVX:	Levofloxacin
MBC:	Minimum bactericidal concentration
MIC:	Minimum inhibitory concentration
ML(V)SS:	Mixed liquor (volatile) suspended solids
NH_4-N:	Ammonia nitrogen
NMDS:	Non-metric multidimensional scaling
NOB:	Nitrite oxidizing bacteria
NO_2-N:	Nitrite nitrogen
NO_3-N:	Nitrate nitrogen
OTUs:	Operational taxonomic units
PO_4-P:	Phosphate phosphorus
SBR:	Sequencing batch reactor
SVI:	Sludge volume index
TN:	Total nitrogen
WWTP:	Wastewater treatment plant

Chapter 1 Introduction

1.1. Antibiotics and their emergence in the environment

The discovery of antibiotics is considered as the most important scientific and medical milestone of the 20th century, however, antibiotics are recently recognized as one emerging kind of environmental contaminants (Carvalho and Santos, 2016).

1.1.1. Antibiotics classification

The classical definition of an antibiotic is a compound produced by a microorganism which inhibits the growth of another microorganism. While, this definition has been expanded to include synthetic and semi-synthetic products over the years.

Antibiotics could be grouped by chemical structure and mechanism of action. They are a diverse group of chemicals which could be divided into different sub-groups (ß-lactams, macrolides, quinolones, sulphonamides, tetracyclines and others) (Kümmerer, 2009). Different antibiotics have various mechanisms of action in attacking the bacteria. Cell membranes, cell-wall biosynthesis enzymes and substrates, bacterial protein synthesis, and bacterial nucleic acid replication and repair were the major targets of antibiotics (Davies and Davies, 2010). Antibiotics could be also classified as causing the death of bacteria (bactericidal) and preventing bacterial growth (bacteriostatic) according to the action modes of antibiotics (Hancock, 2005).

Quinolones play an important role in the treatment of serious bacterial infections. The first and second generation of quinolones (1st generation: nalidixic acid; 2nd generation: e.g. ciprofloxacin, lomefloxacin, norfloxacin, ofloxacin) are active against gram-negative bacteria whereas the third and fourth generation quinolones (3rd generation: e.g. levofloxacin, sparfloxacin, tosufloxacin; 4th generation: e.g. clinafloxacin, gemifloxacin, moxifloxacin, sitafloxacin) have extended activity against gram-positive bacteria as well (Nasuhoglu et al., 2012). Bacteria cannot distinguish such as levofloxacin, ciprofloxacin, norfloxacin and ofloxacin, all of which belong to quinolones, with the same inhibition mechanism for bacterial growth. Thus, in this case, bacteria are exposed to the total quinolone concentrations. In addition, concentrations up to 31 mg/L of nalidixic acid (one kind of the quinolone antibiotics) were measured in the effluent from a large industrial treatment plant, and 45 mg/L of nalidixic acid was detected in the pharmaceutical wastewater (Larsson et al., 2007; Sirtori et al., 2009). Thus, many kinds of quinolone antibiotics could reach wastewater treatment plants (WWTPs) from various sources and they are usually detected ranging from ng/L up to mg/L in the the effluents of hospitals, domestic, and pharmaceutical manufacturing facilities (Larsson et al., 2007; Shi et

al., 2014; Sukul and Spiteller, 2007). Since a variety of new quinolones are commonly found in wastewater matrices, the sum of the biological activities associated with all of these compounds is much higher than the activity of a single compound (Golet et al., 2003; Yang et al., 2008). In a typical wastewater treatment facility, conventional wastewater treatment results in prolonged exposure of the bacteria carried by the wastewater to the quinolones, and the concentrations are significantly higher than the quinolones concentrations present in the effluents. Long-time exposure of bacterial communities to the antibiotics is a condition which could result in the evolution of low-level antibiotic resistance in affected microbial communities (Baquero, 2001; Drlica, 2003).

Levofloxacin (LVX) is a new kind but already widely used quinolone antibiotics (synthetic broad-spectrum antibiotics). It has a wide spectrum of action and requires few doses, which acts by inhibiting bacterial DNA gyrase enzyme required for DNA replication (Kasabe et al., 2009). It has been reported that more than 55 tons of LVX were used for human in USA in 2011 (Yan and Niu, 2017). The levels of LVX measured in the influent at the typical WWTPs in Japan were in the range of 307 to 981 ng/L with the conventional activated sludge process (Yasojima et al., 2006). However, LVX could not be completely metabolized in humans or animals while maintaining its activity, and it also could not be completely removed using current technologies during the wastewater treatment. Thus, eventually, it will be discharged into the environment. New quinolones have been detected in WWTPs, which may have the great effects on organisms and also present a great risk to the human health.

1.1.2. Contributors to antibiotics' emergence in the environment

Antibiotics at natural background concentrations are important for risk assessment of antibiotics, while the anthropogenic input is the main source in the environment (Kümmerer, 2009). A large amount of production and application of antibiotics could enhance the antibiotics dissemination into lots parts of the environments.

(1) Nature source

Most naturally antibiotics are produced by the soil microorganisms. Several antibiotics such as some ß-lactams, aminoglycosides, streptomycins and others could be produced by soil bacteria and fungi. In the soil, the concentrations of antibiotics depend on the density of the antibiotics producers in the environment. In soils and sediments, bacteria are less likely to move than in the free water phase, and the bacterial densities of soils and sediments are higher. Thus the concentrations of naturally-produced antibiotics in the aquatic environment might be even lower, due to the much lower density of microorganisms.

(2) Production and manufacturing

As it was reported that emissions concentrations from production plants in some countries could be several mg/L, which might be found in the effluents for the single compound (Larsson et al., 2007; Li et al., 2008a; Li et al., 2008b). In developed countries, manufacturing plants can also make an important contribution to the total concentrations of antibiotics in the WWTPs influent (Kümmerer, 2009).

(3) Application

It is widely for antibiotics to be used to prevent or treat microbial infections in human and veterinary medicines as well as aquaculture. Hundreds of different kinds of antibiotics or antimycotic substances are applied in human or veterinary medicine (Kümmerer and Henninger, 2003). It has been reported that the consumption of antibiotics worldwide lied 100,000-200,000 tons per annum (Wise, 2002). In America, 50% of the 22,700 metric tons of all antibiotics prescribed each year are for humans, and 50% for agriculture, animals, and fishery products (Kümmerer, 2009).

Human medicine. Consumption for humans in total/per capita/the individual share of each kind of compound is different amoung countries (Kümmerer, 2009). Antibiotic prescription rates and intake without prescription markedly vary amoung countries (Mölstad et al., 2002). The various using levels of a single compound are very common. Depending on the different legislation and the differing using extent of antibiotics, only a few countries provide reliable data on antimicrobial use and per capita consumption patterns. The excretion rate of the unaltered active compound ranges widely (10% to 90%). On average, if the total amount of all antibiotics used is added, the metabolic rate is estimated to be 30%, meaning 70% of them directly discharged into the wastewater unchanged (Kümmerer and Henninger, 2003). Metabolism most often occurs in the liver. In many cases, metabolites are more soluble in water than the parent compound, resulting in excretion of urine. However, occasional metabolite formation may result in the compounds that are more toxic to humans, within the acetylation of sulfamethoxazole as an example (Kümmerer, 2009).

Animals. The consumption of animals for prevention or treatment depends mainly on modern animal breeding and fattening methods and conditions. Antibiotics are commonly used to promote animal growth in some countries where low doses are used in animal feed and are believed to improve the quality of products with lower fat and higher protein content in meat (Gaskins et al., 2002; Cromwell, 2002). The uses of antibiotic growth promoters might impose selective pressure on antibiotic-resistant bacteria in clinical or veterinary practice, thereby

impairing the continued use of antibiotics. Some compounds can be used for the purposes other than human or veterinary, for example, antibiotics could be used for bee-keeping.

Plant agriculture. Antibiotics are mainly used to control certain bacterial diseases of ornamental plants, high-value fruits, and vegetables. The most commonly used antibiotic on plants is streptomycin and oxytetracycline to a minor extent. The main use is apple, pear and related ornamental trees, used for the control of fire blight. Most antimicrobial agents are used to control bacterial diseases of tree fruits. In order to become a strong candidate for disease control, antibiotics must be active on or inside the plants, and need to withstand oxidation, rain, UV irradiation, and high temperatures. On the other hand, these attributes are exactly causing environmental issues.

Aquaculture. Aquaculture is the cultivation of aquatic organisms, with mollusks, fish, crustaceans and aquatic plants included. It means some intervention during the rearing process to enhance production, such as regular feeding, stocking, and protection from predators. In the aquaculture, antibiotics are mainly used for therapeutic purposes and preventive agents (Kümmerer, 2009).

1.2. Antibiotic resistance and its mechanisms

1.2.1. Evolution of antibiotic resistance along with time

Ubiquitous contaminants leading to produce antibiotic resistance bacteria (ARB) and genes (ARGs). Evolution of antibiotic resistance along with time was mainly like followings. In the 1940s, the chemotherapy initiated with some kinds of sulfonamide antibiotics (penicillin, streptomycin, and tetracycline). In the 1950s, a large number of antibiotics were discovered, which are used until today. In the next decade, the amount of the newly discovered antibiotics decreased with the discovery of fluoroquinolones, while the amounts of antibiotic-resistant pathogens increased. In the following years, development of artificial antibiotics was based on the understanding of antibiotics and the resistance, which was owing to the increase of antibiotic-resistant pathogens and the decrease of natural antibiotics discovered. In the 1970s, according to the knowledge of the biochemical actions and resistance mechanisms of antibiotics, artificial modification of the antibiotics chemical structures was carried out to avoid resistance. In the next decade, the genetic researches related to antibiotic targets led to the design of a new class of compounds to avoid antibiotic resistance. In the decade of 1990s, the screening methods were applied to pathogens, used to predict essential targets of antibiotics. A lot of artificial antibiotics were also selected using the high-throughput screening (HTS) assays. From the 2000s till now, Due to the failure of the genome-based discovery programs on antibiotics, lots

of companies disenchanted.

Anthropogenic activities (underutilization, overuse and abuse of antibiotics) have accelerated the development and distribution of antibiotic resistance genes in the microbial community of the whole biosphere (Aminov, 2009; Davies and Davies, 2010).

1.2.2. Antibiotic resistance mechanisms

Mechanism of bacterial resistance to antibiotics could be mainly defined in four main categories as the Figure 1-1 shown: drug inactivation (degradation or modification); action site mutation (qualitative or quantitative); change permeability (inflow inhibition); and drug extrusion by efflux pump. Currently, variations in antibiotic resistance genes in environmental bacteria were identified as evolutionary results of mutations (more than 2 billion years) (Aminov, 2009).

1.3. Fate of antibiotics in WWTPs

1.3.1. The antibiotic residues situation in wastewater treatment plants

WWTPs are widely used as the main collection pools for antibiotics and antibiotic-resistant bacteria, which are increasingly posing a threat to humans (Le-Minh et al., 2010). The detected concentrations of specific antibiotics in raw wastewater are different among countries, possibly reflecting various prescription practices, and the different degrees of dilution because of the differences in water consumption per capita (Le-Minh et al., 2010; Miao et al., 2004).

Antibiotics are often detected in wastewater at concentrations of ng/L and μg/L, which are not sufficient to have a significant impact on wastewater treatment (Le-Minh et al., 2010). However, high concentrations (mg/L) of antibiotics could have an inhibitory effect on the microbial activity of WWTPs. It has been reported that 10-400 mg/L sulfonamide antibiotic could inhibit the microbial activity of activated sludge by 20% or more (Ingerslev and Halling-Sørensen, 2000). Streptomycin (400 mg/L) could inhibit the ammonia oxidation of activated sludge by 75% (Tomlinson et al., 1966). In the anaerobic processes, erythromycin (1 mg/L) can reduce the COD removal rate and biogas production by around 5% (Amin et al., 2006). According to the reports, 6 mg/L of metronidazole can reduce anaerobic activity by about 69% (Gartiser et al., 2007). In addition, sulfamethoxazole and ofloxacin could inhibit methanogenesis during anaerobic digestion processes (Fountoulakis et al., 2004). Ofloxacin can inhibit both the methanogenesis and acetogenic processes (Fountoulakis et al., 2008). Sometimes, complex bacterial mixtures exposed to antibiotics could increase the nitrification activity, however, with no clear reasons (Halling-Sørensen, 2001). The above situations might also reflect the adaptation of microorganisms to antibiotic residues to some extent, which needs

further study.

Traditional WWTPs processes may be variable in the removal of antibiotics. During the anaerobic sludge digestions, the antibiotics adsorbed on the activated sludge can be released into the liquid phase. Therefore, antibiotics might be detected in downstream surface water and groundwater, with the antibiotic resistance increase (Adelowo et al., 2008). In WWTPs, the occurrence, development and transfer of the new resistance gene combinations are more frequently discovered at the higher diversity and microbial density (Bouki et al., 2013). This encourages researchers to study the occurrence and development of antibiotics as well as antibiotic resistance (Le-Minh et al., 2010). However, studies of the causal relationship between antibiotics and resistance gene enhancement have become increasingly difficult and lack reference WWTPs without resistant bacteria and genetic input. In addition, most of the WWTPs are currently contaminated with antibiotic residues. If there is no antibiotic-unexposed WWTPs as a negative control, the effect of antibiotics on the performance of wastewater treatment (C, N and P removal) is very difficult to be discovered, due to the low concentration of antibiotics. In addition, for detection, characterization, quantification both dominant and less dominant but important microorganisms in WWTPs, high throughput methods are necessary, which may help to discover the effects of antibiotics on microbial community shift.

1.3.2. Effect of treatment processes on antibiotics' removal

During the biological treatment, elimination and transformation of antibiotics are the results of various processes, which could be biotic and non-biotic or abiotic. The removal of antibiotics depends to a large extent on the adsorption of the sludge and its degradation or conversion during the processing. Hydrolysis could play an important role for the several compounds, however, photolysis is almost unlikely to occur owing to the low exposure of the material to light during wastewater treatment (Michael et al., 2013).

Hydrophobic antibiotics residues are expected to be the higher concentration in the first and second sludge than hydrophilic sludge (Le-Minh et al., 2010). It is also possible to remove antibiotics from aqueous solutions onto solid particles by ion exchange, complexation with metal ions and polar hydrophilic interactions (Díaz-Cruz et al., 2003). The antibiotics adsorbed in the flocs, suspended solids, and activated sludge could be removed from the aqueous phase by precipitation and subsequent treatment of the remaining sludge. It is worth noting that sludge is often used as a fertilizer in the agricultural sector and can be viewed as another input way for various antibiotics into the environment. It has been reported that ciprofloxacin (quinolone) adsorbs up to 80% onto the sludge, indicating that adsorption is the main removal process

(Michael et al., 2013).

The possible removal of quinolones during conventional wastewater treatment is discussed below. The removal efficiency of norfloxacin and ciprofloxacin (quinolones) in Swedish (during wastewater treatment) was reported to be around 87%, and 86% for ofloxacin (Lindberg et al., 2005). In a later study, ciprofloxacin (> 90%), ofloxacin (56%) and norfloxacin (> 70%) were removed during activated sludge treatment followed by chemical coagulation/aggregation (Zorita et al., 2009). Some authors have already suggested that the main removal mechanism for quinolones is adsorption to sludge and floes rather than biodegradation (Batt et al., 2007; Golet et al., 2003; Lindberg et al., 2006; Zorita et al., 2009). In China, high removals (100%) of ofloxacin has been reported (Peng et al., 2006). The removal efficiency of ciprofloxacin in MBR treating hospital wastewater was only about 51%, might be due to the fact that sludge formation in MBR is lower than that of conventional activated sludge, leading to the lower sorption (Kovalova et al., 2012; Michael et al., 2013).

1.4. Problems statement

Even in the WWTPs with manual enhanced biological treatment process, most antibiotics and their metabolites are permanent in the environment. Persistent antibiotics and derivatives are considered as an ecological factor, inducing phylogenetic structural changes, resistance expansion, and ecological function disturbance of environmental ecology (Ding and He, 2010). In the WWTPs, the discovery of antibiotics is not obvious, because the concentration of antibiotics is low, the microbial mobility is high, and the masking of larger quantities of antibiotic resistance genes and resistant bacteria have been input into the aquatic environment (Ding and He, 2010).

Replacing antibiotic-sensitive bacteria with resistant bacteria may shift the microbial community structures of the WWTPs (Ding and He, 2010; Szczepanowski et al., 2009). An increase in tetracycline-resistant bacteria was found in the SBR exposed to tetracycline (1 mg/L) (Kim et al., 2007). Bacteria resistant to trimethoprim, ciprofloxacin, sulfamethoxazole, and vancomycin have already reported to be found in the influent and effluent of the wastewater (Nagulapally et al., 2009). Most importantly, resistant bacteria might have the similar ecological functions as the substituted sensitive bacteria of complex systems, no matter belonging to the same or different species or not (Ding and He, 2010). Therefore, the choice of resistant bacteria may counteract the inhibition of functional bacteria related to the performance of the WWTPs. Thus, it is very necessary to investigate the microbial community structure shift during wastewater treatment with exposure of antibiotics, in order to shed light on microbial responses

to the antibiotic.

Many researches have been focused on antibiotics contamination, but most of the antibiotics are made by microorganisms. That is why the antibiotics from human activity are hard to analyze. So, we focus on quinolones that is completely synthetic antibiotics. No degrader and producer of quinolones live in the environment as so far. The adaptation mechanisms of bacteria community exposed to acute and chronic/fluctuated LVX levels during wastewater treatment in SBRs need to be investigated.

1.5. Research objectives and thesis structure

(1) To do the risk assessment of LVX resistance/tolerance with the sludge samples taken from WWTPs after synthetic wastewater domestication;

(2) To better understand the performance changes of wastewater treatment with the acute LVX exposure operation in SBRs;

(3) To investigate the effects of chronic/fluctuated LVX on the performance of the wastewater treatment in SBRs;

(4) To quantify the dynamic changes of microbial communities exposed to the antibiotic for adaptation to residual antibiotics leading to recovered functions of wastewater treatment.

In the first part of this study (Chapter 2), the resistance pattern test (MIC and MBC) of the sludge samples taken from the four SBRs was performed before the LVX exposure operation. The data is useful for further exposure operation concentrations' deciding. In the second and third part of the study (Chapter 3 and Chapter 4), the better understanding of the effects of acute and chronic/fluctuated LVX on the performance of the wastewater treatment in SBRs was focused in this study. In the fourth part of this study (Chapter 5), in addition to the changes in the performance of the wastewater treatment, the dynamic changes of microbial communities will be also quantified to further investigate the adaptation mechanisms of bacteria community.

The whole structure of this thesis is illustrated in Figure 1-2.

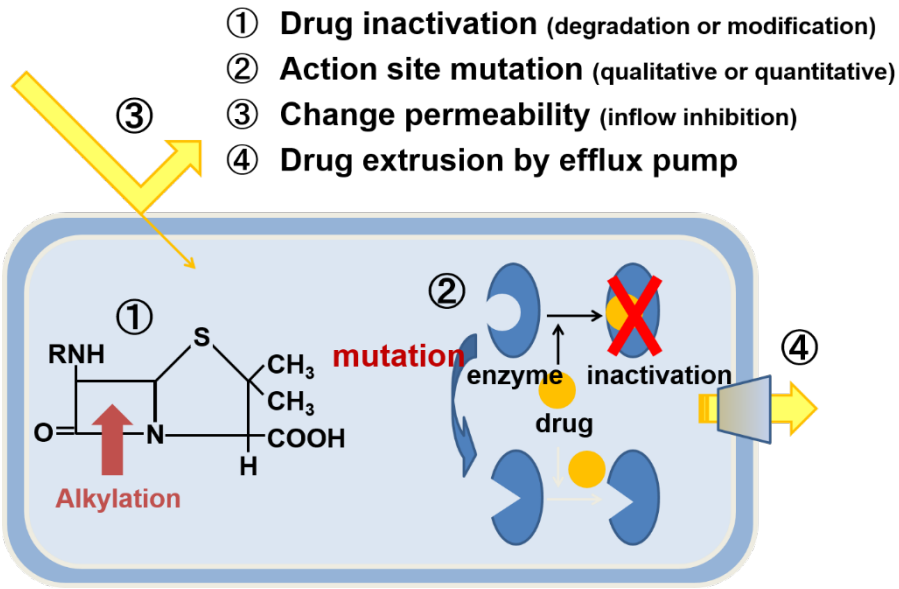

Figure 1-1 Schematic diagram of four main antibiotic resistance mechanisms.

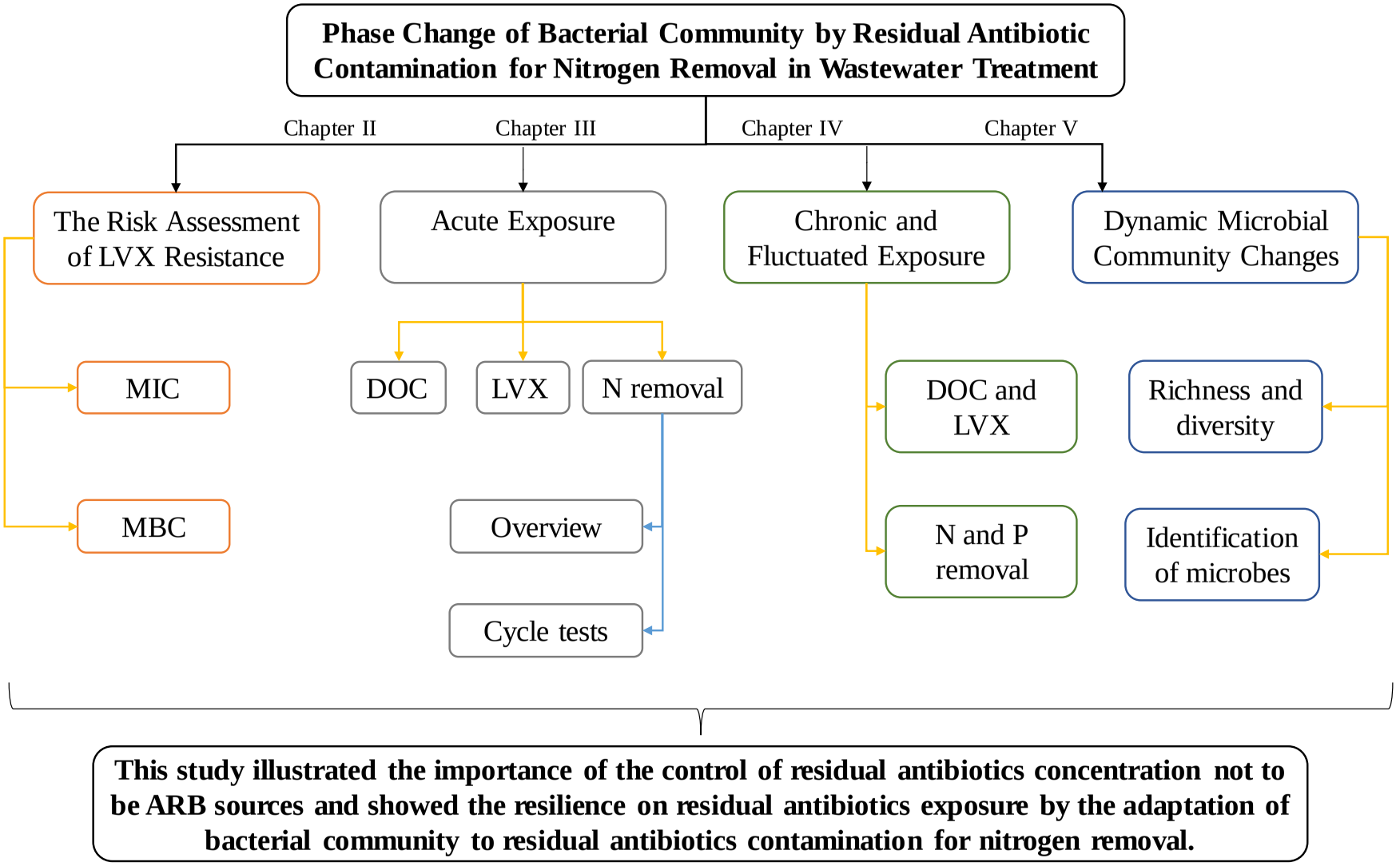

Figure 1-2 Research route and framework of this thesis.

Chapter 2 The Risk Assessment of LVX Resistance

2.1. Introduction

Most of the antibiotics with their metabolites are persistent in the environments, even in the biological treatment processes of WWTPs, the main collection pools of antibiotics and antibiotic-resistant bacteria. Wastewater that contains antibiotic residues are observed worrisome because of antibiotics could exert selective pressure and might contribute to the appearance of resistant bacteria. The concentration of specific antibiotics in wastewater varies from country to country, however, persistent antibiotics and their metabolites could be thought as ecological factors (inducing phylogenetic structure alteration, resistance expansion, and ecological function disturbance of environmental ecology). Bacterial resistance to antibiotics can uncover many molecular mechanisms of resistance, such as drug inactivation (degradation or modification), action site mutation (qualitative or quantitative), change permeability (inflow inhibition) and drug efflux by efflux pump. It is worth noting that the bacterial resistance/tolerance mechanisms could decrease the effectiveness of antibiotics, which means that high concentrations of the antibiotic are required to produce the same effect in a resistant strain as is produced in a susceptible strain.

In practice, the minimum inhibitory concentration (MIC) is measured by exposing the bacterial population to an increasing concentration of antibiotics using a standardized growth medium. The MIC determined by these tests is considered as a convenient measure of resistance, and bacterial strains with higher MIC than the other strain are considered to be more resistant (Scholar and Pratt, 2000). Furthermore, in the clinic, the simplicity of measuring MIC means that it is the only standard currently used to inform treatment decisions for bacterial strains isolated (Paterson et al., 2001). Minimum bactericidal concentration (MBC) generally indicates the concentration of antibiotic required to kill ≥99.9% of cells in the bacterial culture after 24 hours of incubation. The concentration of antibiotics near the MIC only causes growth arrest, however, the MBC results in death.

After domestication with the synthetic wastewater for around 5 months, sludge samples taken from the four SBRs were used to perform the resistance pattern test (MIC and MBC) like below. The results of this chapter could reveal the already existing antibiotic resistance level of the sludge taken from WWTPs using in this study.

2.2. Materials and methods

2.2.1. Experimental set-up and operation conditions

Four identical SBRs were used in this study, made of acrylic plastic. The height was 50 cm, with the inner diameter of 3.6 cm and an effective working volume of 366 ml for each reactor. As shown in Figure 2-1, during the aeration period, the air pump (AK-30, KOSHIN, Japan) was used with the air bubble diffusers at the bottom of each SBR reactor (0.75 cm/s of the air flow rate). During aeration, the dissolved oxygen (DO) was at 8-10 mg/L. In the study, synthetic wastewater was used with its composition shown as follows (per liter): 191.07 mg NH_4Cl, 384.62 mg CH_3COONa, 100 mg $NaHCO_3$, 21.94 mg KH_2PO_4, 51.25 mg $MgSO_4•7H_2O$, 36.75 mg $CaCl_2•2H_2O$, 21.82 mg $FeSO_4•7H_2O$ and 1 mL trace element solution. The trace metals solution consisted of (per liter): $AlCl_3$ (50 mg), $CoCl_2•6H_2O$ (50 mg), $CuCl_2$ (30 mg), H_3BO_3 (50 mg), $MnSO_4•H_2O$ (50 mg), $(NH_4)_6Mo_7O_{24}•4H_2O$ (50 mg), $NiCl_2$ (50 mg) and $ZnCl_2$ (50 mg). The quality of levofloxacin ($C_{18}H_{20}N_3O_4F$, >98%) was guaranteed by Tokyo Chemical Industry, Japan. The LVX solution (40 g-LVX/L) was prepared in 0.1 M HCl solution before the time of use and then add to the synthetic wastewaters. Each SBR was inoculated with 100 mL of sludge before starting the acclimation by synthetic wastewater. The initial mixed liquor suspended solids (MLSS) concentration was 3.5 g/L. The sludge volume index (SVI) was 100.9 mL/g with MLVSS/MLSS of 0.8 in the four SBRs. All the reactors were operated sequentially in a 6 h cycle at room temperature (25 ± 2°C): influent filling (15 min), non-aeration period (60 min), aeration (240 min), settling (30 min), and effluent discharge (15 min). The volumetric exchange ratio was kept at 53%, with a hydraulic retention time (HRT) of 11.3 h.

2.2.2. Minimum inhibitory concentration

MIC was determined by using the plates containing LVX as the Figure 2-2 shown. This procedure involved preparing two-fold dilutions of LVX (eg, 0, 0.125, 0.25, 0.5, 1, and 2 mg/L) in the synthetic wastewater-gellan gum growth medium dispensed in plates. The antibiotic-containing plates were inoculated with the 0.1 mL sludge sample. Following 24 h incubation at 25°C under dark condition, the colonies were examined for visible bacterial growth. The lowest concentration of antibiotic that prevented growth after overnight incubation represented the MIC.

2.2.3. Minimum bactericidal concentration

MBC was determined from MIC tests by sub-culturing to synthetic wastewater-gellan gum plates that with and without LVX as the Figure 2-3 shown. The MBC was identified by determining the lowest LVX concentration that reduces the viability of the initial bacterial inoculum by ≥99.9%.

2.3. Results and discussion

2.3.1. Minimum inhibitory concentration profiles of LVX

To assess the exposure effect on tolerant/resistant of bacteria in reactors, samples taken from the four reactor systems were used to perform the resistance pattern test before the LVX exposure operation (Table 2-1 and Table 2-2). For this kind of exposure antibiotic of LVX, the bacteriostatic effect on all bacteria was confirmed at the concentration of 2,048 mg-LVX/L due to LVX functions which are inhibition of DNA gyrase and production of radicals to kill the bacteria (Kohanski et al., 2007). Concentrations below 32 mg-LVX/L didn't show any obvious bacteriostatic action for the sludge samples taken from the four reactors. The MICs for all the four reactors were determined as 32 mg-LVX/L.

2.3.2. Minimum bactericidal concentration profiles of LVX

MBC is a supplement to the MIC and the MIC test showed the lowest level of antibiotics inhibited the growth, while MBC showed the lowest level of antibiotics that caused microbial death. In this study, the MBCs for all four reactors (R1, R2, R3, and R4) were 512 mg-LVX/L. The results of MIC (32 mg-LVX/L) and MBC (512 mg-LVX/L) of the sampled sludge reflect that the LVX resistance/tolerance has already existed in the actual WWTPs. The LVX concentration levels of the directly filtered sampled sludge were in the range of 0.09 to 0.76 mg-LVX/L in this study. It has been also reported that the levels of LVX measured in the influent at the typical WWTPs in Japan were in the range of 307 to 981 ng/L with the conventional activated sludge process (Yasojima et al., 2006).

According to the above results, the exposure concentrations of LVX in R2, R3 and R4 were decided for Stage I. R1, the control reactor, was fed with synthetic wastewater only. R2 was used to treat with synthetic wastewater containing 4 mg-LVX/L (lower than the MIC concentration). R3 and R4 were fed with synthetic wastewater containing 16 mg-LVX/L (half of the MIC concentration) and 128 mg-LVX/L (the quarter of the MBC concentration), respectively.

2.4. Summary

(1) The related results of LVX resistance for sludge samples taken from SBRs have been shown in Table 2-1 and Table 2-2.

(2) The MICs of sludge samples for all four reactors are 32 mg-LVX/L.

(3) The MBCs of samples for all four reactors are 512 mg-LVX/L.

(4) Acute LVX exposure concentrations (Chapter 3) were decided according to the results of MIC and MBC above.

Table 2-1 The MIC of LVX for microbial community before exposure operation.

LVX concentration (mg/L)	R1	R2	R3	R4
2048	s	s	s	s
1024	s	s	s	s
512	s	s	s	s
256	s	s	s	s
128	s	s	s	s
64	s	s	s	s
32	s	s	s	s
16	+	+	+	+
8	+	+	+	+
4	+	+	+	+
2	+	+	+	+
1	+	+	+	+
0.5	+	+	+	+
0.25	+	+	+	+
0.125	+	+	+	+
Control ($\times 10^6$ cfu/mL)	2.13±0.32	2.63±0.06	2.50±0.44	2.03±0.42

(+: overserved bacterial growth as colony; s: no observed bacterial growth)

Table 2-2 The MBC of LVX for microbial community before exposure operation.

(a) First step results:

LVX concentration (mg/L)	R1	R2	R3	R4
2048	s	s	s	s
1024	s	s	s	s
512	s	s	s	s
256	s	s	s	s
128	s	s	s	s
64	s	s	s	s
32	s	s	s	s
16	s	s	s	s
Control ($\times 10^6$ cfu/mL)	2.26±0.12	2.28±0.06	2.36±0.32	2.16±0.23

(b) Second step results for MBC determination:

LVX concentration (mg/L)	**With LVX**				**Without LVX**			
	R1	R2	R3	R4	R1	R2	R3	R4
2048	s	s	s	s	s	s	s	s
1024	s	s	s	s	s	s	s	s
512	s	s	s	s	s	s	s	s
256	s	s	s	s	+	+	+	+
128	s	s	s	s	+	+	+	+
64	s	s	s	s	+	+	+	+
32	s	s	s	s	+	+	+	+
16	+	+	+	+	+	+	+	+

(+: overserved bacterial growth as colony; s: no observed bacterial growth)

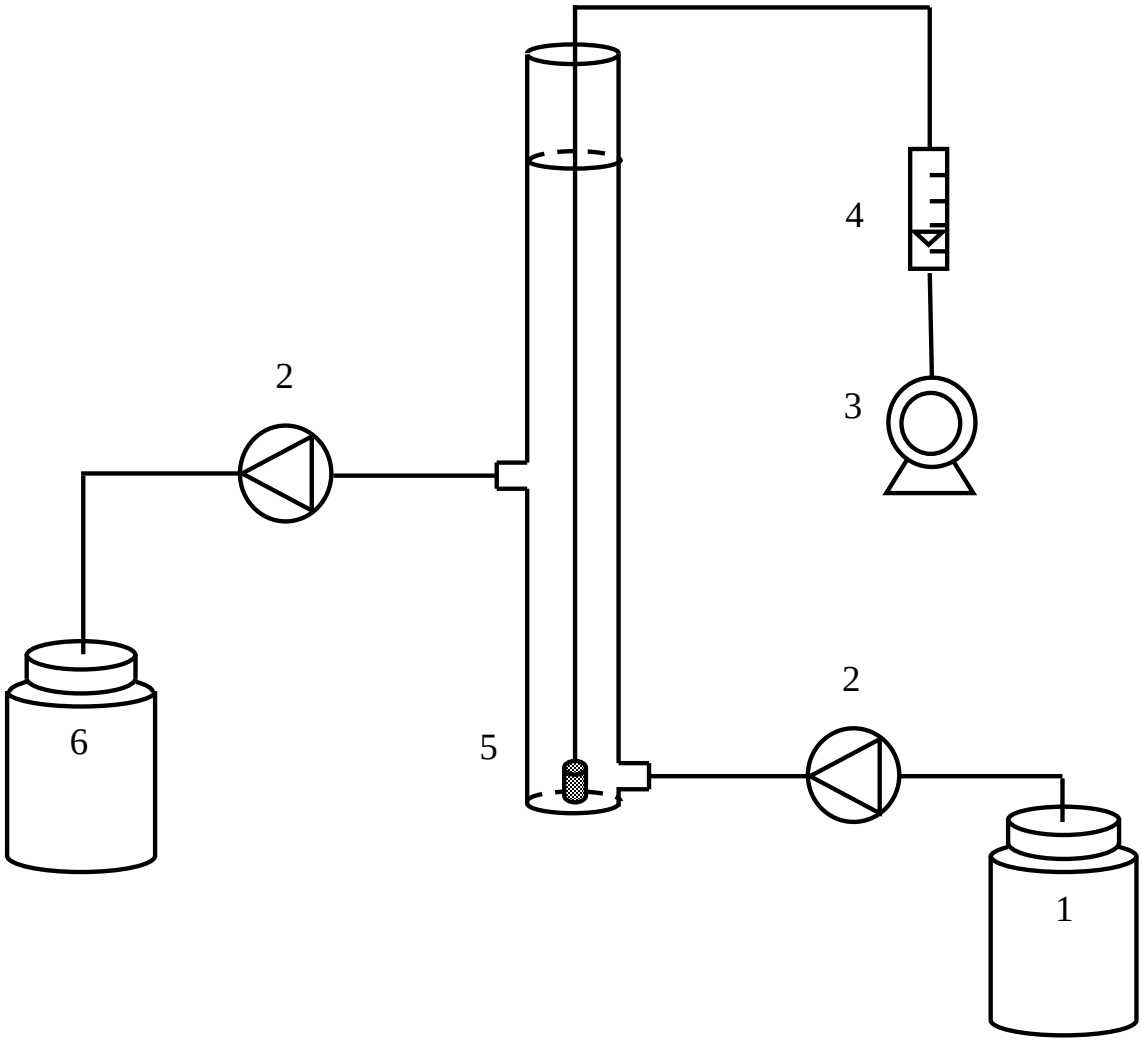

Figure 2-1 Experimental apparatus for SBR employed in present study.

1-Influent, 2-Water pump, 3-Air pump, 4-Flowmeter, 5-Aeration line, 6-Effluent.

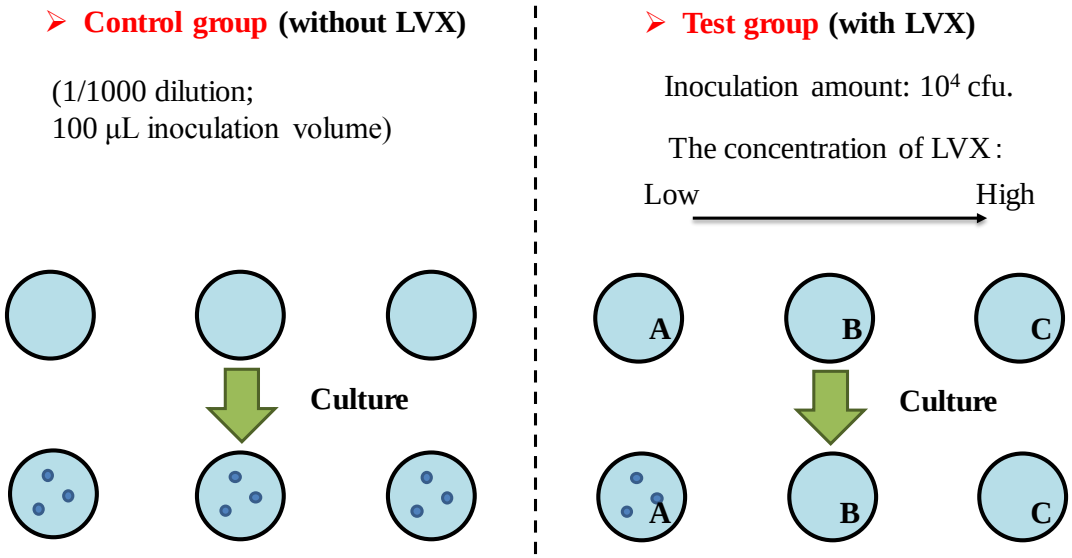

Figure 2-2 MIC determination method.

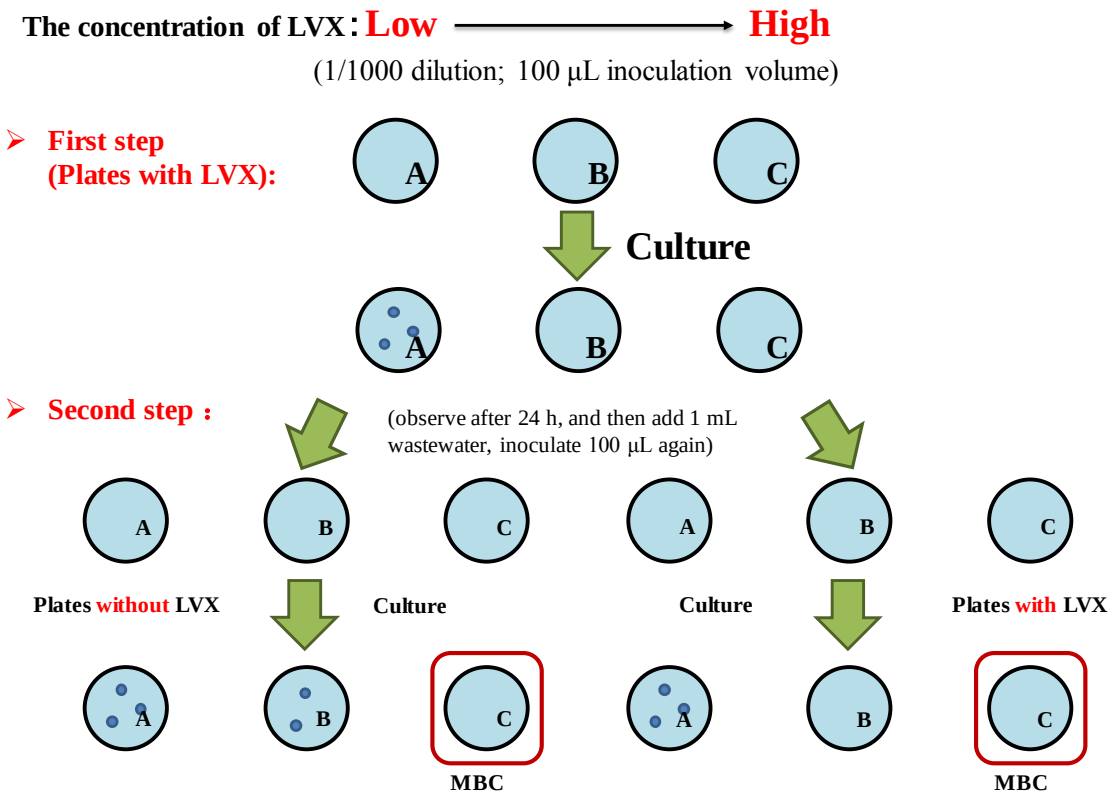

Figure 2-3 MBC determination method.

Chapter 3 Acute LVX Exposure

3.1. Introduction

The development of antibiotics generates large amounts of antibiotic residues in the environment each year, which has become a widespread concern in recent years. Human drugs are mainly discharged through domestic wastewater (the mixture of feces, urine, and water) in the sewer network (Alighardashi et al., 2009). It will place a heavy burden on the environment if wastewater treatment is inadequate, and the manufacturing facilities may also be an important source of supply (Larsson et al., 2007). The outflow of agricultural facilities is another cause of antibiotic contamination (Zheng et al., 2018).

One of the manmade antibiotics, quinolones, are very important in the treatment of serious bacterial infections. Quinolones (typically from ng/L to mg/L) could arrive at WWTPs from different pathways, such as the effluents of hospitals, domestic, and pharmaceutical manufacturing facilities (Larsson et al., 2007; Sukul and Spiteller, 2007). In India, nalidixic acid (quinolone antibiotics) with a concentration of up to 31 mg/L has been reported in the wastewater from the large industrial treatment plants (Larsson et al., 2007). In addition, 45 mg/L of nalidixic acid has been reported to be measured in the pharmaceutical wastewater by Sirtori et al. (2009).

LVX is a new kind but already widely used quinolone antibiotics, the most utilization in Japan. Further according to the U.S. outpatient prescription data, 11.3 million LVX prescriptions were used in 2014, increasing by 21.5% when compared to those in 2010 (Bidell and Lodise, 2016). As the last barrier before LVX enters the aquatic environment, the WWTPs are manipulated to culture a range of microorganisms for removing contaminants. In addition, current WWTPs are not specifically designed to remove these complex and persistent compounds. The LVX might also be toxic to the engineered microorganisms, thus, reducing the performance of WWTPs. For this reason, the feasibility of comprehensive exploring the acute exposure of LVX is demanding to avoid the release of the antibiotic residues into the environment, which are possibly in drinking or reclaimed water.

To date, however, it has not been reported about the potential acute toxicity of LVX to wastewater nitrogen removal. The aim of this chapter is to shed light on better understanding the changes in the performance of wastewater treatment with acute LVX exposure. To the best of our knowledge, this is the first study fully evaluating the potential acute toxicity of LVX to wastewater treatment process, and the findings obtained here appeal engineers to pay more

attention to the toxicity of these emerging contaminants such as LVX to wastewater biological treatment processes. The findings from this work are expected to provide good guidance on performance recovery of WWTPs when encountering accidental loading of antibiotics like LVX.

3.2. Materials and methods

3.2.1. Experimental set-up and operation conditions

Four identical SBRs were used in this study, made of acrylic plastic. The height was 50 cm, with the inner diameter of 3.6 cm and an effective working volume of 366 ml for each reactor. During the aeration period, the air pump (AK-30, KOSHIN, Japan) was used with the air bubble diffusers at the bottom of each SBR reactor (0.75 cm/s of the air flow rate). During aeration, the dissolved oxygen (DO) was at 8-10 mg/L. In the study, synthetic wastewater was used with its composition shown as follows (per liter): 191.07 mg NH_4Cl, 384.62 mg CH_3COONa, 100 mg $NaHCO_3$, 21.94 mg KH_2PO_4, 51.25 mg $MgSO_4•7H_2O$, 36.75 mg $CaCl_2•2H_2O$, 21.82 mg $FeSO_4•7H_2O$ and 1 mL trace element solution. The trace metals solution consisted of (per liter): $AlCl_3$ (50 mg), $CoCl_2•6H_2O$ (50 mg), $CuCl_2$ (30 mg), H_3BO_3 (50 mg), $MnSO_4•H_2O$ (50 mg), $(NH_4)_6Mo_7O_{24}•4H_2O$ (50 mg), $NiCl_2$ (50 mg) and $ZnCl_2$ (50 mg). The quality of levofloxacin ($C_{18}H_{20}N_3O_4F$, >98%) was guaranteed by Tokyo Chemical Industry, Japan. The LVX solution (40 g-LVX/L) was prepared in 0.1 M HCl solution before the time of use and then add to the synthetic wastewaters. Each SBR was inoculated with 100 mL of sludge before starting the acclimation by synthetic wastewater. The initial mixed liquor suspended solids (MLSS) concentration was 3.5 g/L. The sludge volume index (SVI) was 100.9 mL/g with MLVSS/MLSS of 0.8 in the four SBRs. All the reactors were operated sequentially in a 6 h cycle at room temperature (25 ± 2°C): influent filling (15 min), non-aeration period (60 min), aeration (240 min), settling (30 min), and effluent discharge (15 min). The volumetric exchange ratio was kept at 53%, with a hydraulic retention time (HRT) of 11.3 h. These SBRs were under the dark condition for avoiding light.

The operation was conducted as follows. R1 was the control reactor fed with synthetic wastewater only, and the remaining 3 reactors were operated as tested reactors: R2 was used for treating synthetic wastewater containing 4 mg-LVX/L, and R3 and R4 were fed with synthetic wastewater containing 16 mg-LVX/L and 128 mg-LVX/L, respectively.

3.2.2. Chemical and physical analysis

Influent and effluent samples were collected once every day and then filtered through 0.22 μm membrane prior to analysis. The parameters related to SBR performance (ammonia nitrogen

(NH_4-N), nitrite nitrogen (NO_2-N), nitrate nitrogen (NO_3-N) and total nitrogen (TN) concentrations) in addition to mixed liquor (volatile) suspended solids (ML(V)SS), and sludge volume index (SVI) were analyzed according to standard methods (APHA, 2012). TOC analyzer ($TOCV_{CSN}$, SHIMADZU, Japan) equipped with an auto-sampler (ASI-V, SHIMADZU, Japan) was used to measure the dissolved organic carbon (DOC). Dissolved oxygen concentration was analyzed by DO meter (HQ40d, HACH, USA) in the reactors and pH was monitored using a pH meter (Horiba, Japan).

LVX concentrations in the filtrates were determined by a high-performance liquid chromatography (HPLC) system (JASCO, Japan) equipped with an LC-Net II/ADC system controller, UV-1570 intelligent UV/VIS detector, PU-1580 intelligent HPLC pumps, CO-1560 intelligent column thermostat and AS-1555-10 intelligent sampler. Separation was carried out on a 5C18-AR-II column (4.6 mm×150 mm) at 30°C and the flow rate was controlled at 1 mL/min (mobile phase) with an injection volume of 20μL. Briefly, the mobile phase contained a mixture of 0.025 mol/L phosphate buffer at pH 3.0 (80%) and acetonitrile (20%) (Sun et al., 2018). Detection was carried out at a wavelength of 294 nm. All the determinations were performed in triplicate, and average values were used if there's no special indication.

3.3. Results and discussion

3.3.1. DOC removal performance

After about 5 months' domestication (before acute exposure), all the four SBR reactors have shown a stable performance with average effluent DOCs from R1, R2, R3 and R4 being around 5.5 mg/L, 6.1 mg/L, 7.0 mg/L and 6.1 mg/L, respectively (Figure 3-1). Results show that the four SBRs can be used to effectively remove organics from wastewater.

During acute exposure, with the addition of LVX, results (Figure 3-1) show that the effluent DOC has shown extremely high in R4, around 105 mg/L. This phenomenon can be attributable to the following two aspects. On the one hand, LVX ($C_{18}H_{20}N_3O_4F$) contains carbon, and 128 mg-LVX/L could add about 77 mg/L DOC more into the influent. Thus, the difference between 105 mg/L and 77 mg/L (or 28 mg/L) might the actual effluent DOC (excluding adsorption effect and the DOC caused by LVX) from R4, which is still much greater than its performance before the Stage I (average effluent DOC ~8.2mg/L). This observation suggests that LVX could inhibit the uptake and degradation of organics by the microorganisms due to antibiotic functions which are inhibition of DNA gyrase and production of radicals to kill the bacteria (Kohanski et al., 2007).

3.3.2. Changes in LVX concentrations during the acute exposure

The LVX concentration of the directly filtered inoculated sludge samples were in the range of 0.09 to 0.76 mg-LVX/L in this study. During acute exposure, with the addition of LVX, results (Figure 3-2) show that the effluent LVX concentrations levels. Like other antibiotics (Alighardashi et al., 2009), partial LVX might be adsorbed onto the biomass due to its very low biodegradability. From the Figure 3-2, still, at least half of the influent LVX was left in the effluent, meaning that LVX could not be completely removed during wastewater treatment using the current process like SBR. Thus, along with the effluent discharge to the environment, LVX would be transferred to other environmental compartments, spreading the problem to many related ecosystems.

3.3.3. Nitrogen removal profiles

(1) Overall performance during acute LVX exposure operation

As seen, the four SBRs exhibited excellent performance in treating NH_4-N wastewater with NH_4-N removal rate > 99% and excellent nitrification efficiency (99-100%) (Figure 3-7). Results show that all the four systems exhibited almost similar efficiencies in overall nutrients removals, implying their excellent stability in SBRs before the acute LVX exposure.

During the acute LVX exposure, NH_4-N removals in R3 and R4 were remarkably affected (Figure 3-3). Especially in R4 exposed to 128 mg-LVX/L, a large amount of NH_4-N was still detected in its effluent, most probably the inhibition of ammonia-oxidizing bacteria (AOB) by the high concentration of LVX resulting in much lower ammonia oxidation efficiency (Figure 3-7). The specific ammonia uptake rate was detected to decrease with the increase in influent LVX concentration, thus ammonia accumulation occurred. After some adaptation during Stage I, NH_4-N in R3 and R4 recovered. From the picture of Figure 3-4 the increase in effluent NO_2-N concentration signaled the recovery of nitrification in R3 and R4. The accumulation of NO_2-N might be associated with the inhibition both on denitrification and nitratation (from NO_2-N to NO_3-N) processes. Correspondingly, it was also noticed that the exposure of LVX exerted the notable effect on the effluent NO_3-N concentration (Figure 3-5). Yi et al. (2017) claimed that the activity of nitrite reductase could be inhibited by the presence of ciprofloxacin, one kind of new quinolones as same as LVX. During the exposure test, effluent TN concentrations increased, especially from R4. This observation might not only be attributable to inhibited denitrification, but also the addition of LVX ($C_{18}H_{20}N_3O_4F$) which contains 11.7% of N (w/w). It has been reported before that the NO_3-N concentration obtained with the addition of antibiotic depended upon the balance between nitrification inhibition (which reduces the final concentration) and cell lysis (which increases the nitrogen pool in terms of ammonia and

organic nitrogen, thus increasing the final concentration) (Alighardashi et al., 2009).

(2) Nitrogen removal profiles during cycle tests

The variations of pH, DO and DOC in the bulk liquor during cycle tests before the acute exposure were shown in Figure 3-8. The variation of N concentrations in the bulk liquor was also monitored in the four reactors during typical cycle tests (like before acute LVX exposure). Results (Figure 3-9a) showed that more than 99% of influent NH_4-N could be removed within 360 min in all SBRs. Similarly, during a typical operation cycle before acute LVX exposure, results of N profiles (Figure 3-9) showed the consistently similar trend in all four reactors.

After around 25 d LVX exposure, a typical operation cycle (Figure 3-10) was chosen to show more detail information. Still, more than 99% of influent NH_4-N could be removed at the end of 360 min (Figure 3-10a). However, clearly higher NH_4-N was observed in R4 and R3 (Figure 3-10a) during the cycle processes. Figure 3-10b showed that during one cycle, more NO_2-N accumulated in R4 and R3, accordingly, more NO_3-N concentrations monitored in R1 and R2 (Figure 3-10c). The TN concentrations of effluent from R4 were detected higher obviously than R1 (Figure 3-10d), as reported previously that some antibiotics could inhibit important microbial processes like denitrification (Costanzo et al., 2005; Yi et al., 2017).

3.4. Summary

(1) During acute LVX exposure, results show that the effluent DOC has shown extremely high in R4, around 105 mg/L. This phenomenon can be attributable to the two aspects: 1) LVX ($C_{18}H_{20}N_3O_4F$) contains carbon, and 128 mg-LVX/L could add about 77 mg/L DOC more into the influent. 2) This observation suggests that LVX could inhibit the uptake and degradation of organics by the microorganisms due to antibiotic functions which are inhibition of DNA gyrase and production of radicals to kill the bacteria.

(2) Partial LVX might be adsorbed onto the biomass due to its very low biodegradability. Still, at least half of the influent LVX was left in the effluent, meaning that LVX could not be completely removed during wastewater treatment using the current process like SBR. Thus, along with the effluent discharge to the environment, LVX would be transferred to other environmental compartments, spreading the problem to many related ecosystems.

(3) NH_4-N removals in R3 and R4 were remarkably affected. Especially in R4 exposed to 128 mg-LVX/L, a large amount of NH_4-N was still detected in its effluent, most probably due to the inhibition of AOB by the high concentration of LVX resulting in much lower ammonia oxidation efficiency. The specific ammonia uptake rate was detected to decrease with the increase in influent LVX concentration, thus ammonia accumulation occurred. After some

adaptation, NH_4-N in R3 and R4 recovered.

(4) The increase in effluent NO_2-N concentration signaled the recovery of nitrification in R3 and R4. The accumulation of NO_2-N might be associated with the inhibition both on denitrification and nitratation (from NO_2-N to NO_3-N) processes.

(5) The exposure of acute LVX exerted the notable effect on the effluent NO_3-N concentration. This might be because the activity of nitrite reductase could be inhibited by the presence of LVX.

(6) During the acute LVX exposure, effluent TN concentrations increased, especially from R4. This observation might not only be attributable to inhibited denitrification, but also the addition of LVX ($C_{18}H_{20}N_3O_4F$) which contains 11.7% of N (w/w).

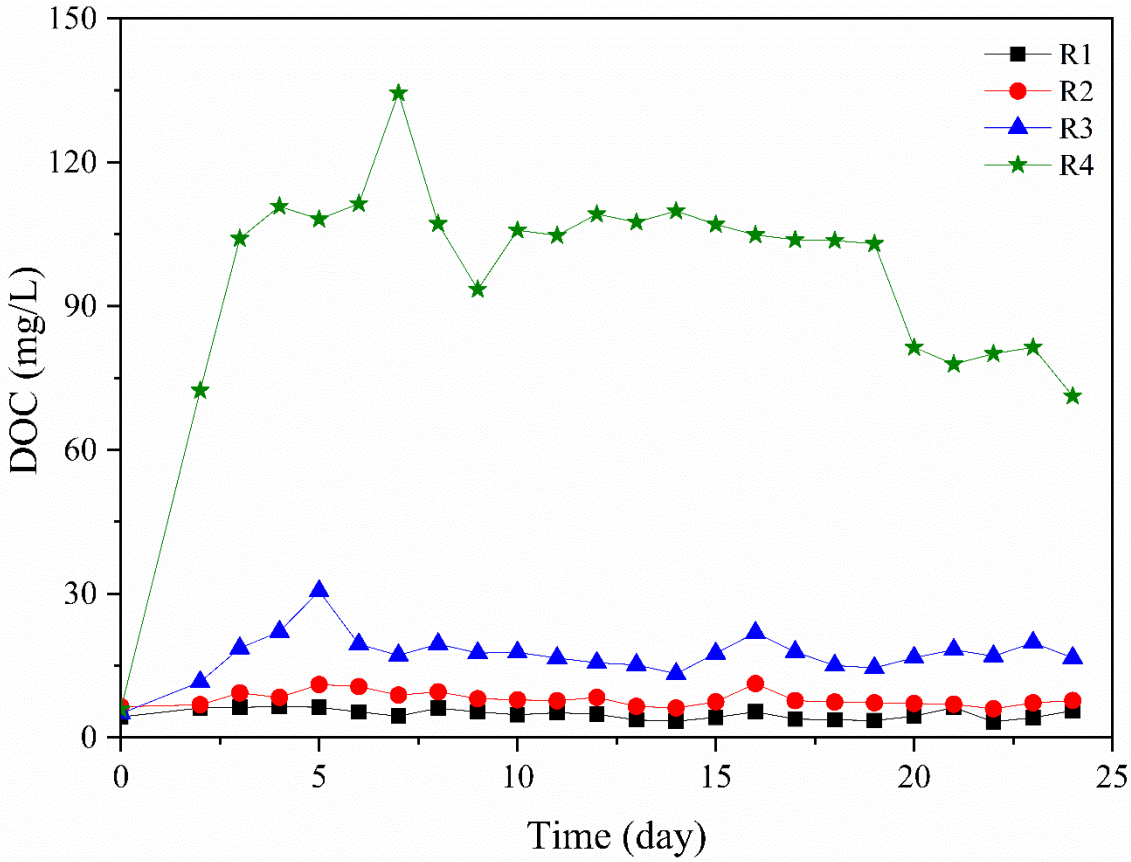

Figure 3-1 DOC concentration changes during acute LVX exposure operation. R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L).

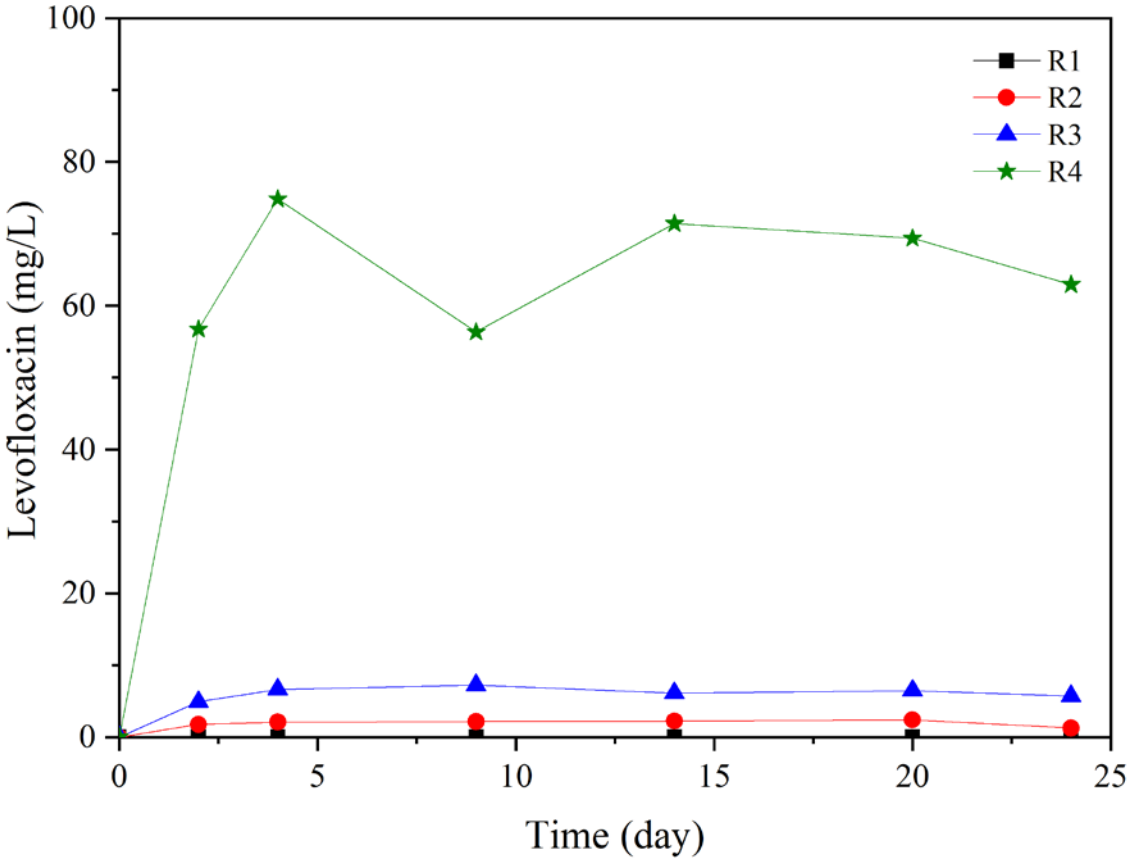

Figure 3-2 LVX concentration changes during acute LVX exposure operation.
R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L).

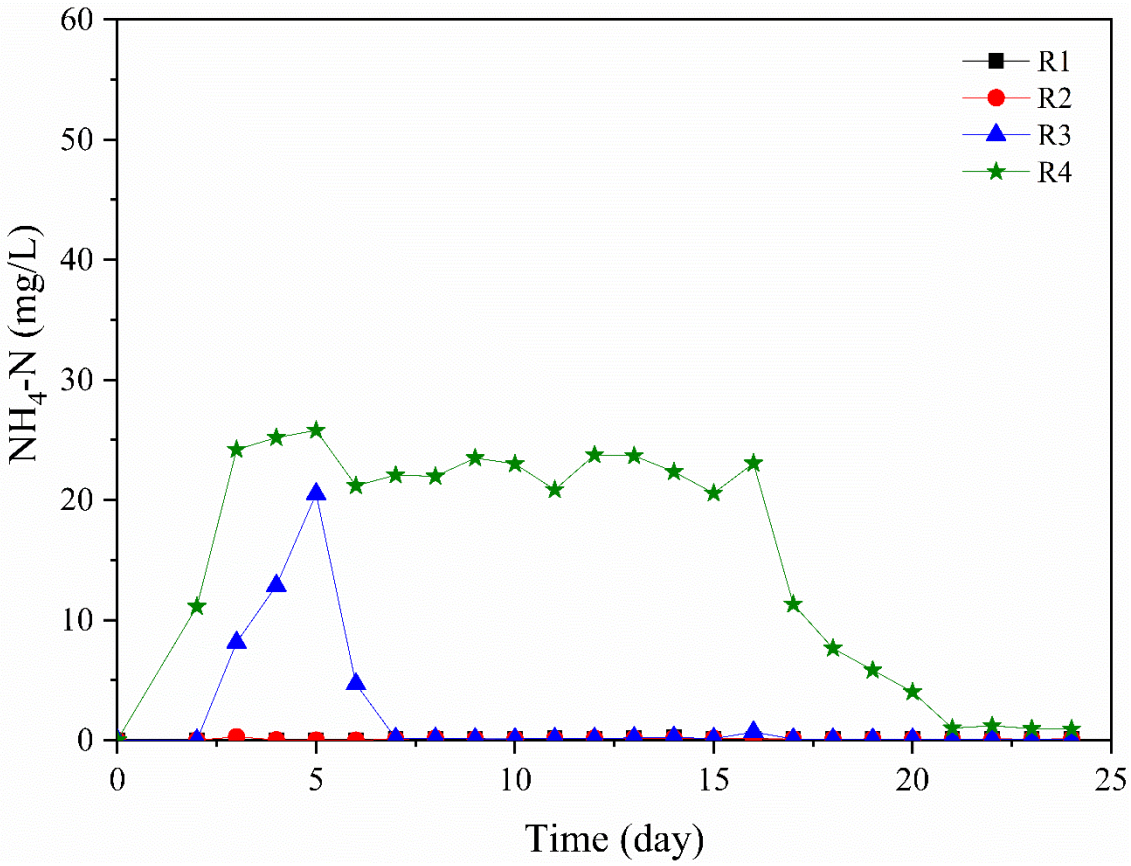

Figure 3-3 NH_4-N concentration changes during acute LVX exposure operation. R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L).

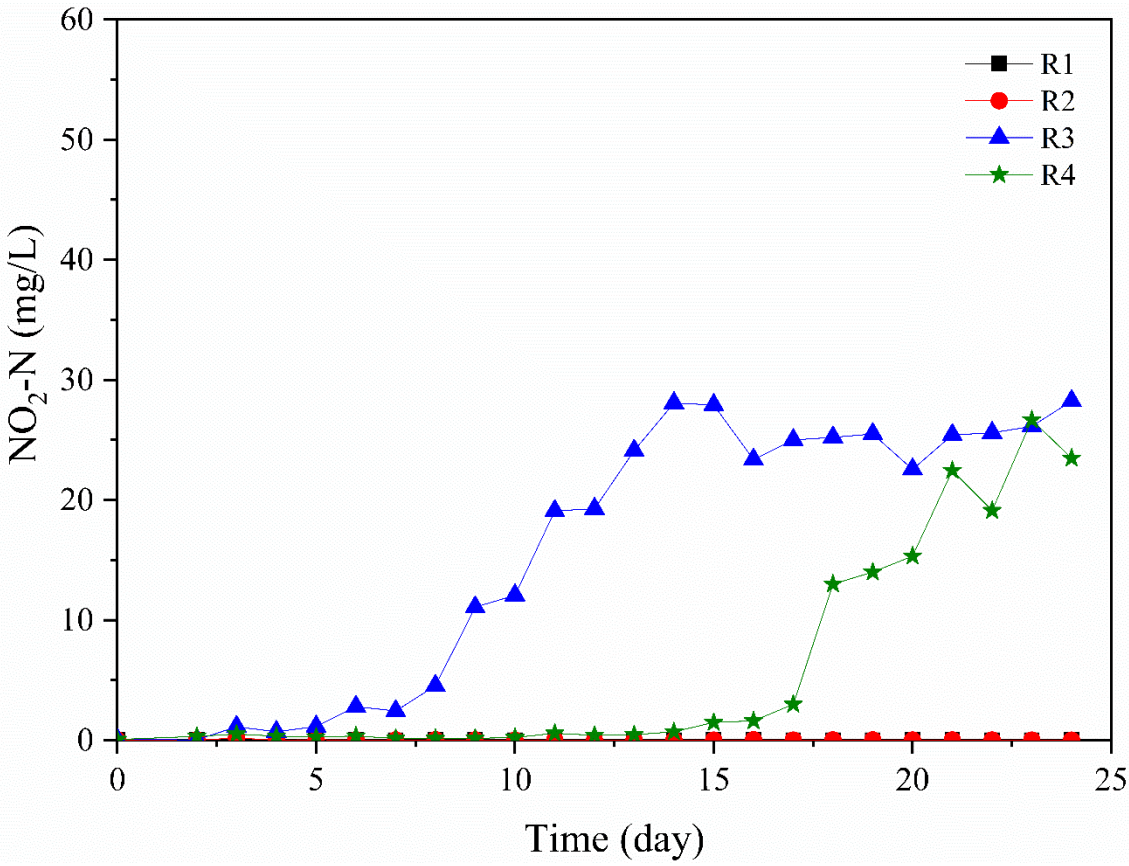

Figure 3-4 NO_2-N concentration changes during acute LVX exposure operation. R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L).

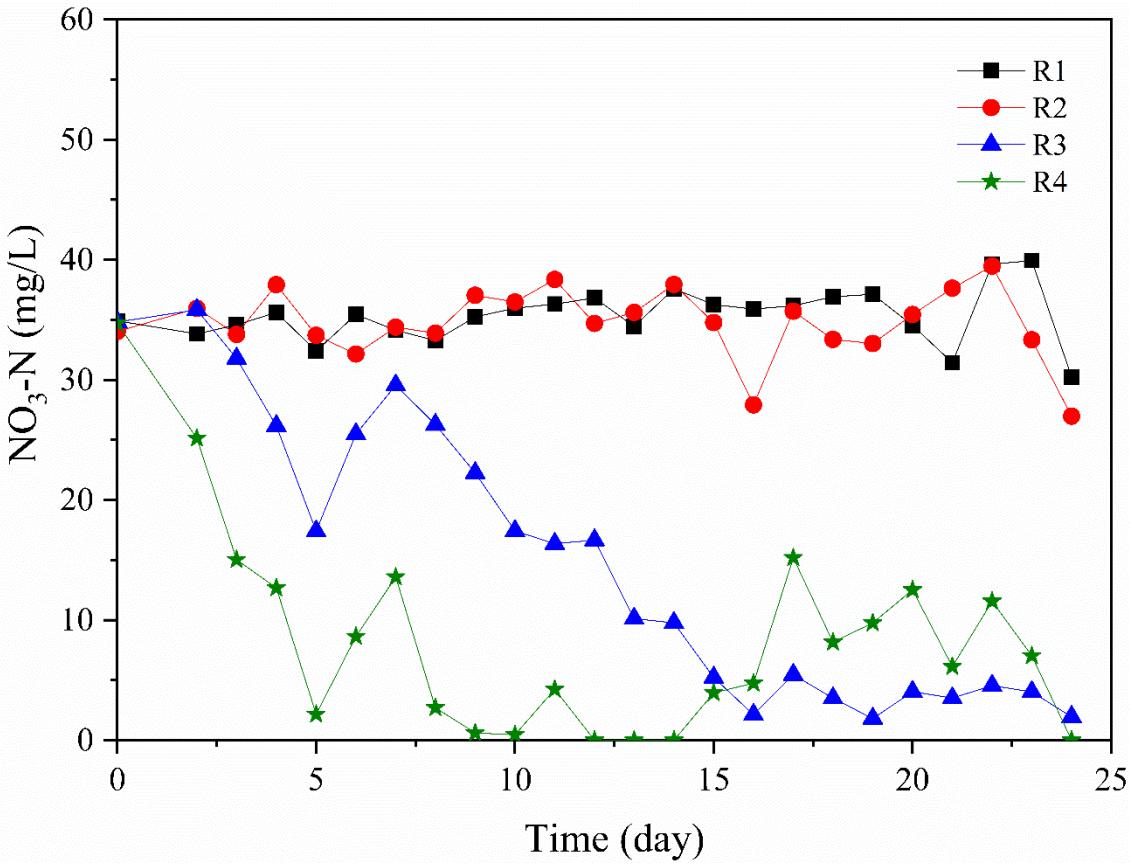

Figure 3-5 NO_3-N concentration changes during acute LVX exposure operation. R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L).

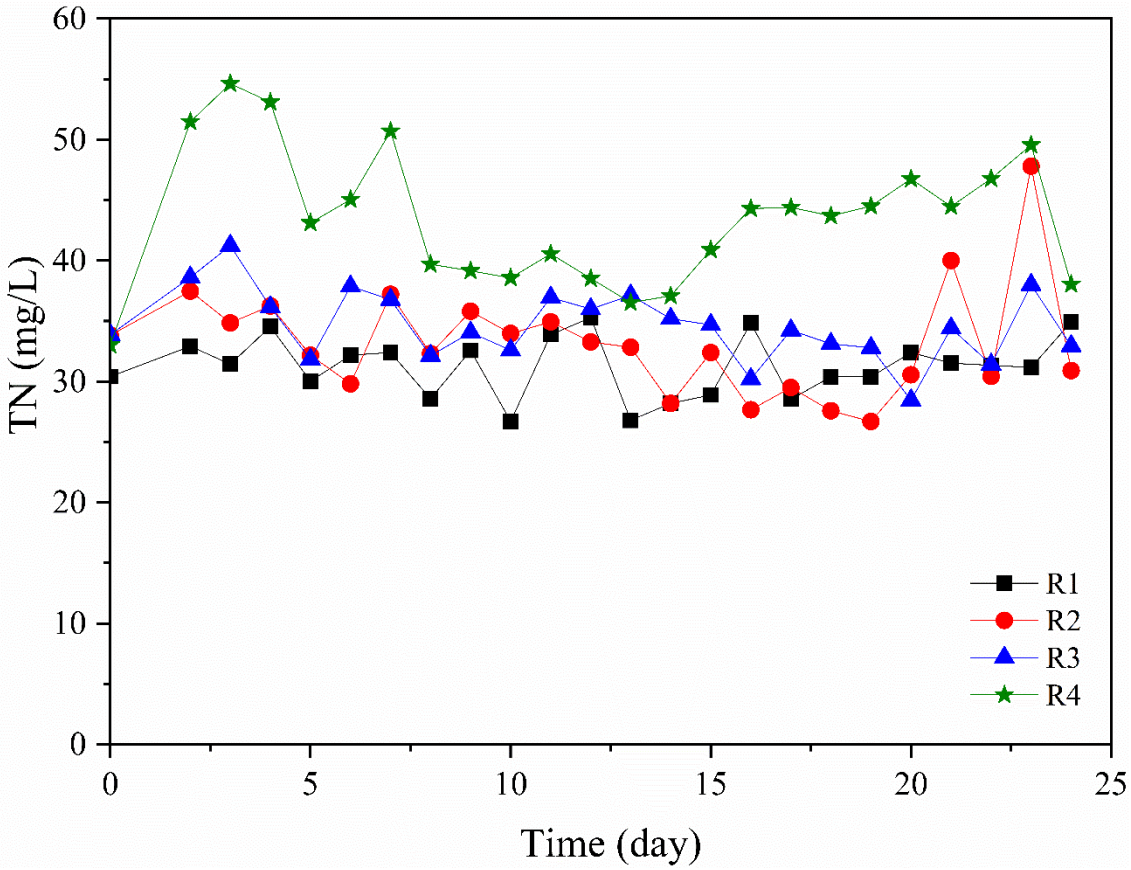

Figure 3-6 TN concentration changes during acute LVX exposure operation.

R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L).

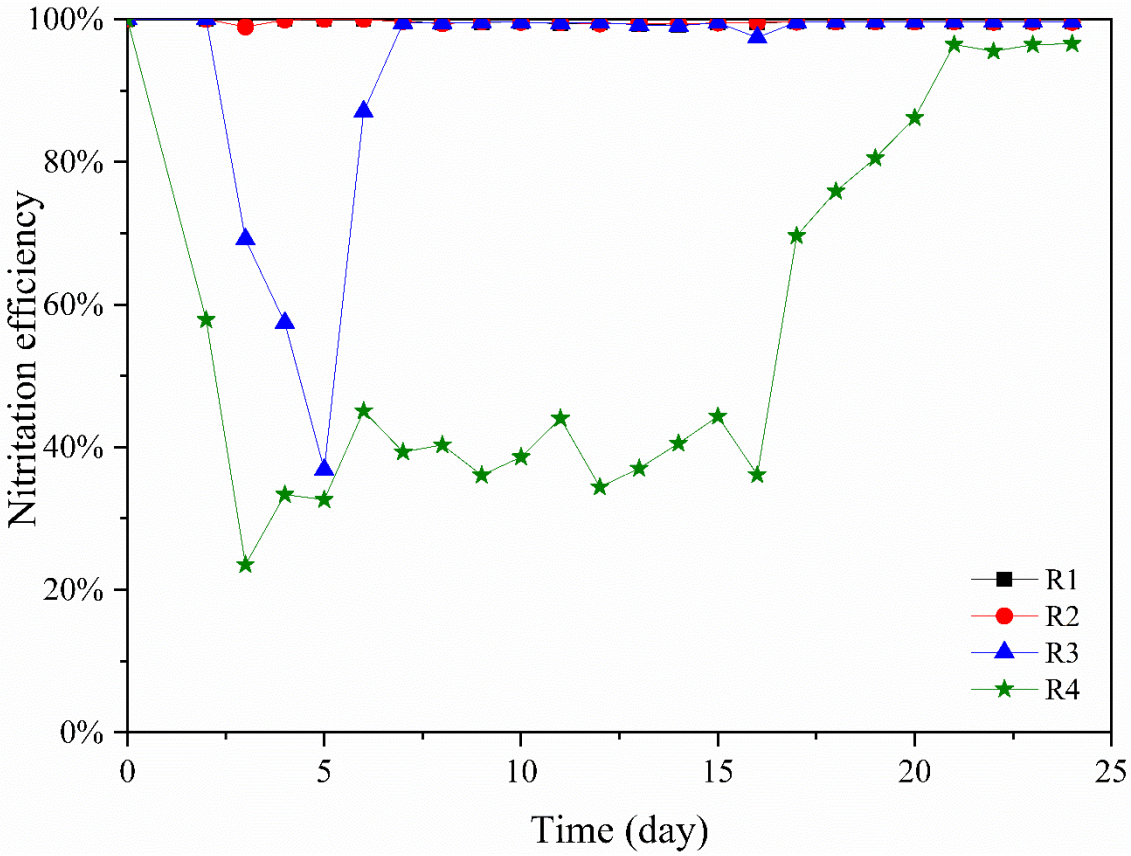

Figure 3-7 Nitritation efficiency changes during acute LVX exposure operation. R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L).

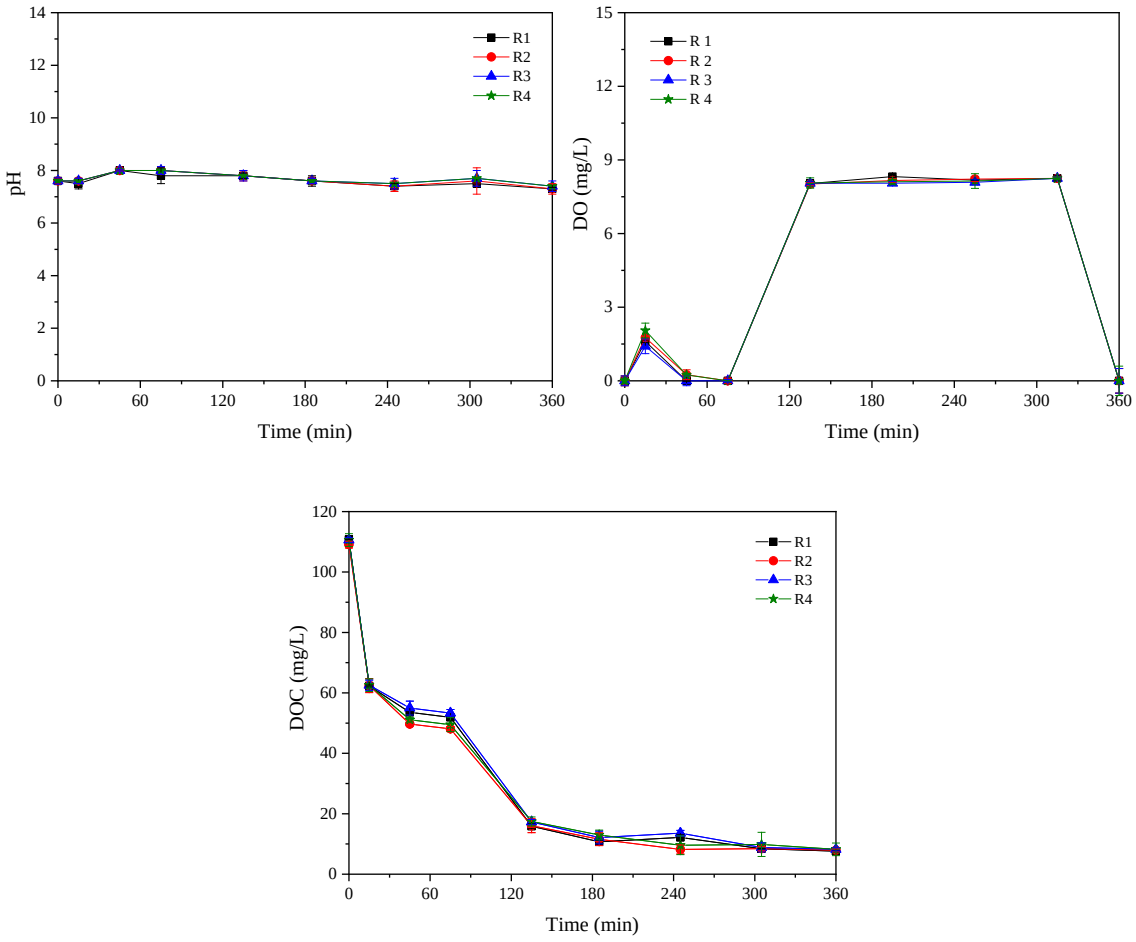

Figure 3-8 Variations of pH, DO and DOC in the bulk liquor during cycle tests before the acute exposure.

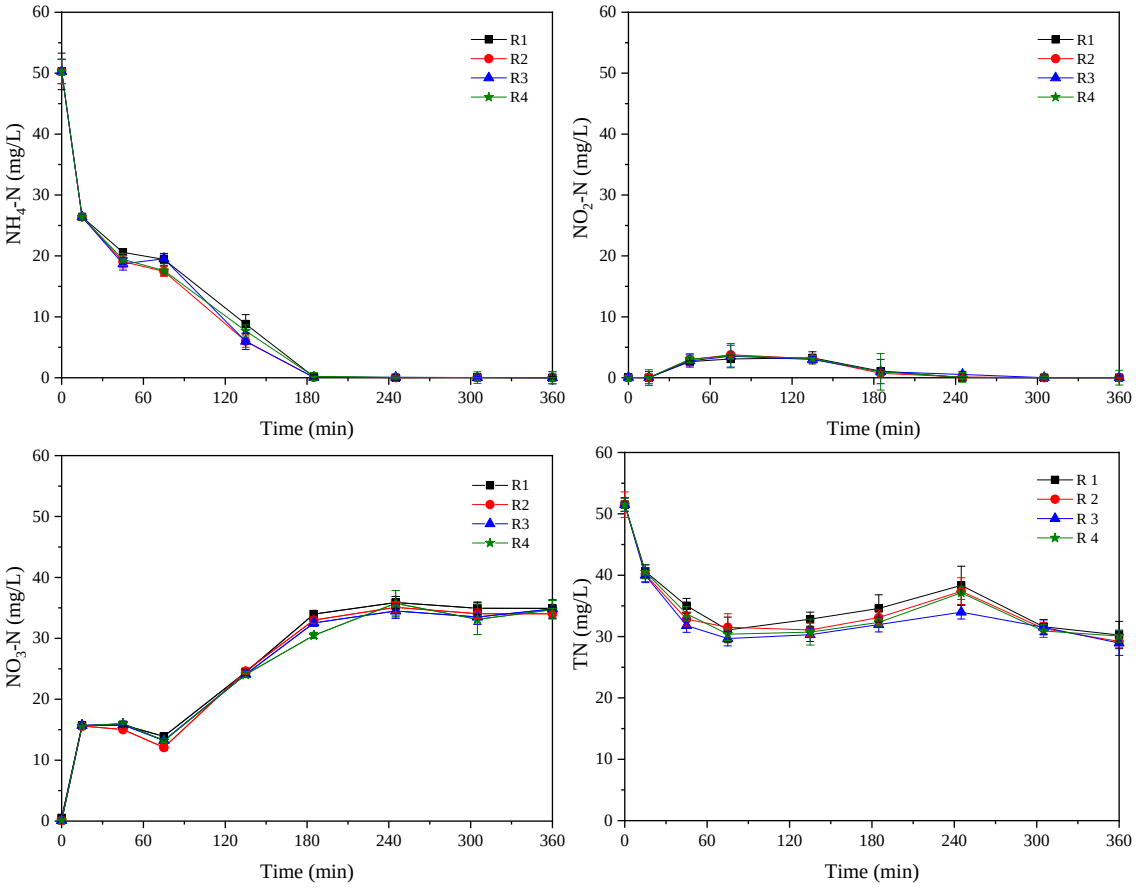

Figure 3-9 Variations of NH_4-N, NO_2-N, NO_3-N and TN in the bulk liquor during cycle tests before the acute exposure.

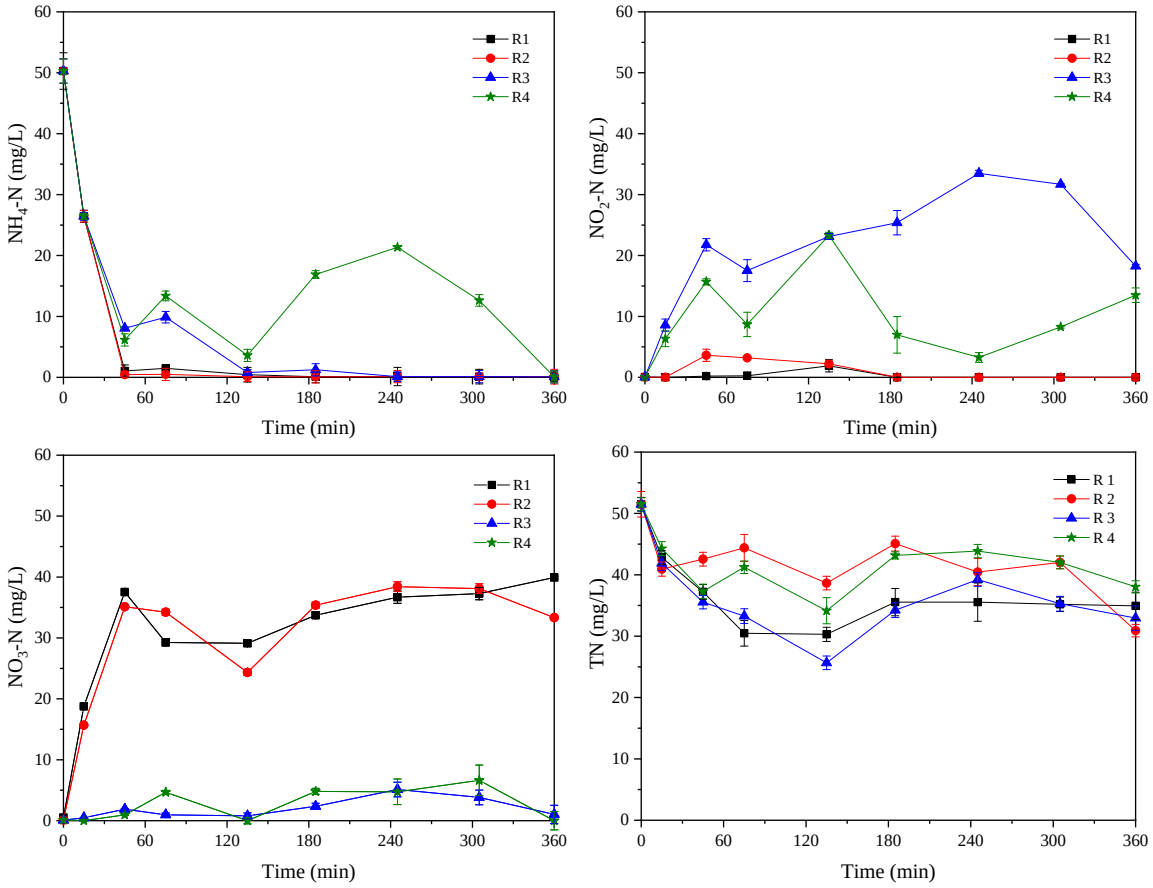

Figure 3-10 Variations of NH_4-N, NO_2-N, NO_3-N and TN in the bulk liquor during cycle tests after the acute exposure.

Chapter 4 Chronic and Fluctuated LVX Exposure

4.1. Introduction

A study of 76 countries between year 2000 and 2015 found that antibiotics consumption, expressed in defined daily doses (DDD), increased by 65% (21.1-34.8 billion DDDs), resulting in antibiotics consumption rate increased by 39% (11.3-15.7 DDDs per 1,000 inhabitants per day) (Klein et al., 2018). Nowadays antibiotics are ubiquitous environmental contaminants discharged from pharmaceutical factories, medical facilities, animal husbandry and domestic sources. In aquatic ecosystems, the consistent input of antibiotics leads to widespread distribution, occurrence and development of antibiotic residues (Kümmerer, 2009; Yan et al., 2013). One frequently detected class of synthetic antibiotics, quinolones, are very important during the treatment of serious bacterial infections. It has a wide spectrum of action and requires a few doses, which acts by inhibiting bacterial DNA gyrase enzyme required for DNA replication (Kasabe et al., 2009). Bacteria cannot distinguish such as levofloxacin, ciprofloxacin, norfloxacin and ofloxacin, all of which belong to quinolones, with the same inhibition mechanism for bacterial growth. Thus, in this case, bacteria are exposed to the total quinolone concentrations. In addition, concentrations up to 31 mg/L of nalidixic acid (one kind of the quinolone antibiotics) were measured in the effluent from a large industrial treatment plant, and 45 mg/L of nalidixic acid was detected in the pharmaceutical wastewater (Larsson et al., 2007; Sirtori et al., 2009). Thus, many kinds of quinolone antibiotics (typically from ng/L to mg/L) could arrive at WWTPs from different pathways, such as the effluents of hospitals, domestic, and pharmaceutical manufacturing facilities (Larsson et al., 2007; Shi et al., 2014; Sukul and Spiteller, 2007).

Levofloxacin (LVX), a new quinolone type and widely used quinolone antibiotics, is mostly utilized in Japan. According to the U.S. outpatient prescription data, 11.3 million LVX prescriptions were dispensed in 2014, increasing by 21.5% when compared to those in 2010 (Bidell and Lodise, 2016). In addition, LVX could not be completely metabolized in humans and animals while maintaining its activity, which also could not be completely removed during wastewater treatment with the current technologies. Thus, after being utilized, it would be discharged into the environment eventually and transferred to other environmental compartments, spreading this problem to many other related ecosystems. More seriously, it has been reported that low levels of multiresistant bacteria and ARGs were detected 3.2 km from the discharge pipe of the WWTP, where lake water was used for drinking water production

(Czekalski et al., 2012). Although the discharge of wastewater to Vidy Bay results in more ARG accumulation in sediments near the contamination source, the diffusion zone of elevated ARG concentrations extends further west towards the location of the drinking water pumping station, which indicated that transportation happens over a considerable distance (Czekalski et al., 2014). Also, the WWTPs are mainly operated without the final disinfection units, and most of them rely on the conventional biological treatment in Switzerland and many other European countries according to Czekalski et al. (2014). Importantly, the reason why most WWTPs look like working well till now even though lots of antibiotic residues might already exist in the wastewater needs to be explored. In addition, up to now, very little information could be found on how microbial community adapts when exposed to the residual antibiotics.

In this work, sequencing batch reactor (SBR) was used as it has been regarded as one of the promising biotechnologies in wastewater treatment due to its incomparable advantages including single-tank configuration, small foot print, easily expandable, simple operation and low capital costs (Mahvi, 2008). To mimic the uncontrolled or accidental discharge of LVX to the WWTPs, both exposure and re-exposure of varying LVX concentrations were considered and designed in the experiments. This study aimed to shed light on the acclimation mechanisms of bacteria to fluctuated LVX levels in SBRs. The effects of LVX exposure/re-exposure on nutrients removal from the wastewater were also recorded and analyzed.

4.2. Materials and methods

4.2.1. Experimental set-up and operation conditions

Four identical SBRs were used in this study, made of acrylic plastic. The height was 50 cm, with the inner diameter of 3.6 cm and an effective working volume of 366 ml for each reactor. During the aeration period, the air pump (AK-30, KOSHIN, Japan) was used with the air bubble diffusers at the bottom of each SBR reactor (0.75 cm/s of the air flow rate). During aeration, the dissolved oxygen (DO) was at 8-10 mg/L. In the study, synthetic wastewater was used with its composition shown as follows (per liter): 191.07 mg NH_4Cl, 384.62 mg CH_3COONa, 100 mg $NaHCO_3$, 21.94 mg KH_2PO_4, 51.25 mg $MgSO_4•7H_2O$, 36.75 mg $CaCl_2•2H_2O$, 21.82 mg $FeSO_4•7H_2O$ and 1 mL trace element solution. The trace metals solution consisted of (per liter): $AlCl_3$ (50 mg), $CoCl_2•6H_2O$ (50 mg), $CuCl_2$ (30 mg), H_3BO_3 (50 mg), $MnSO_4•H_2O$ (50 mg), $(NH_4)_6Mo_7O_{24}•4H_2O$ (50 mg), $NiCl_2$ (50 mg) and $ZnCl_2$ (50 mg). The quality of levofloxacin ($C_{18}H_{20}N_3O_4F$, >98%) was guaranteed by Tokyo Chemical Industry, Japan. The LVX solution (40 g-LVX/L) was prepared in 0.1 M HCl solution before the time of use and then add to the synthetic wastewaters. Each SBR was inoculated with 100 mL of sludge

before starting the acclimation by synthetic wastewater. The initial mixed liquor suspended solids (MLSS) concentration was 3.5 g/L. The sludge volume index (SVI) was 100.9 mL/g with MLVSS/MLSS of 0.8 in the four SBRs. All the SBRs were operated in a 6 h cycle at room temperature (25 ± 2°C): influent filling (15 min), non-aeration period (60 min), aeration (240 min), settling (30 min), and effluent discharge (15 min). The volumetric exchange ratio was kept at 53%, with a hydraulic retention time (HRT) of 11.3 h. These SBRs were under the dark condition for avoiding light.

The operation was conducted in 5 stages (I, II, III, IV and V) for totally around 185 days Table 4-1. The conditions for each stage were as follows. (1) Stage I was the first time's exposure of LVX to the bacteria. R1 was the control reactor fed with synthetic wastewater only, and the remaining 3 reactors were operated as tested reactors: R2 was used for treating synthetic wastewater containing 4 mg-LVX/L, and R3 and R4 were fed with synthetic wastewater containing 16 mg-LVX/L and 128 mg-LVX/L, respectively. (2) Stage II was started after around 25 days' exposure experiment. The LVX exposure was stopped and all the 4 reactors were fed with synthetic wastewater with no antibiotics about 25 days to recover. (3) Stage III lasted about 74 days, during which only synthetic wastewater was fed to the 4 reactors. (4) A 25 days' LVX re-exposure experiment was performed in Stage IV: R1 was still operated as the control fed with the same synthetic wastewater as above, while R2 and R3 were changed to treat synthetic wastewater containing 128 mg-LVX/L, and R4 was fed with synthetic wastewater containing 4 mg-LVX/L. (5) Stage V: After 25 days' re-exposure experiment, all the 4 reactors were again fed with synthetic wastewater only for 25 days to recover their nutrients removal performance.

4.2.2. Chemical and physical analysis

Influent and effluent samples were collected once every day and then filtered through 0.22 μm membrane prior to analysis. The parameters related to SBR performance (phosphate phosphorus (PO_4-P), ammonia nitrogen (NH_4-N), nitrite nitrogen (NO_2-N), nitrate nitrogen (NO_3-N) and total nitrogen (TN) concentrations) in addition to mixed liquor (volatile) suspended solids (ML(V)SS), and sludge volume index (SVI) were analyzed according to the standard methods (APHA, 2012). TOC analyzer ($TOCV_{CSN}$, SHIMADZU, Japan) equipped with an auto-sampler (ASI-V, SHIMADZU, Japan) was used to measure the dissolved organic carbon (DOC). Dissolved oxygen concentration was analyzed by DO meter (HQ40d, HACH, USA) in the reactors and pH was monitored using a pH meter (Horiba, Japan).

LVX concentrations in the filtrates were determined using a high-performance liquid

chromatography (HPLC) system (JASCO, Japan) equipped with an LC-Net II/ADC system controller, UV-1570 intelligent UV/VIS detector, PU-1580 intelligent HPLC pumps, CO-1560 intelligent column thermostat and AS-1555-10 intelligent sampler. Separation was carried out on a 5C18-AR-II column (4.6 mm×150 mm) at 30°C and the flow rate was controlled at 1 mL/min (mobile phase) with an injection volume of 20μL. Briefly, the mobile phase contained a mixture of 0.025 mol/L phosphate buffer at pH 3.0 (80%) and acetonitrile (20%) (Sun et al., 2018). Detection was carried out at a wavelength of 294 nm. All the determinations were performed in triplicate, and average values were used if there's no special indication.

4.3. Results and discussion

4.3.1. DOC removal and its relationship with LVX concentrations applied

After about 5 months' acclimation (before Stage I), the four SBRs can be used to effectively remove organics from wastewater shown as Figure 4-1. The variations of pollutants concentrations in the bulk liquor were also monitored in the four reactors during a typical operation cycle before Stage I. During Stage I, with the addition of LVX, the effluent DOC from R4 increased sharply, averagely around 105 mg/L. This observation suggests that LVX could inhibit the uptake and degradation of organics by the microorganisms. Seen from Figure 4-2, still, at least half of the influent LVX was left in the effluent, which could not be completely removed during wastewater treatment using the current process like SBR.

After stopping the exposure to LVX (Stage II), both R3 and R4 recovered organics removal efficiency quickly, and after 25 days' operation with no LVX in the influent, all the 4 reactors exhibited a stably excellent performance for DOC removal (Figure 4-1). In addition, the effluent LVX concentrations from R2, R3 and R4 were below 1 mg/L (Figure 4-2). During Stage III, all the 4 reactors were operated continuously for about 74 days by feeding the synthetic wastewater only (without LVX), purposefully to confirm their performance recovery and then the LVX re-exposure experiment started (Stage IV). During Stage III, all SBR reactors showed recovered and stable performance on DOC removal, achieving average effluent DOC around 8 mg/L (Figure 4-1). This observation indicates that SBRs could be used to effectively remove organics again, even after being exposed to LVX for 25 days.

In this study, for the first time the re-exposure experiment was conducted after the recovery from the LVX exposure during Stage I, which is probably the actual situation of WWTPs all over the world. During the re-exposure experiments, the LVX concentrations applied to each reactor was changed to further investigate the bacterial adaptation when exposed to fluctuated LVX concentrations. During Stage IV, with the addition of LVX again, better DOC removal

performance was achieved in R2 and R3 when compared to R4 during Stage I (they were fed with the same concentration of LVX, 128 mg/L, Figure 4-1). Similarly, results from Stage IV indicate that LVX could inhibit the uptake of organics by the microorganisms. Interestingly, a better DOC removal performance was noticed in R2 (exposed to 4 mg-LVX/L in Stage I while 128 mg-LVX/L in Stage IV) than in R3 (exposed to 16 mg-LVX/L in Stage I while 128 mg-LVX/L in Stage IV) during the re-exposure test. However, their effluent LVX concentrations were similar during Stage IV, in accordance with those from R4 during Stage I.

Again, after stopping the re-exposure experiments by feeding the synthetic wastewater only (Stage V), all the four reactors rapidly recovered their performance in terms of DOC removal.

4.3.2. Nitrogen and phosphorus removal profiles

As seen, the four SBRs exhibited excellent performance in treating NH_4-N wastewater with NH_4-N removal rate > 99% and excellent nitrification efficiency (99-100%). In this work, the influent phosphorus in synthetic wastewater was prepared with KH_2PO_4, thus TP removal can be reflected by PO_4-P removal. Results show that the effluent PO_4-P concentrations from R1 to R4 were averagely 1.9 mg/L, 2.0 mg/L, 1.8 mg/L, and 1.9 mg/L, respectively (Figure 4-3). No significant difference in TP removal efficiency (45%-48%) was detected among R1, R2, R3 and R4. Results show that all the four SBRs exhibited almost similar efficiencies in overall P removal, implying the excellent stability in P removal by using SBR.

During the LVX exposure in Stage I, NH_4-N removals in R3 and R4 were remarkably affected (Figure 4-4). Especially in R4 exposed to 128 mg-LVX/L, a large amount of NH_4-N was still detected in its effluent, most probably the inhibition of AOB by the high concentration of LVX resulting in much lower ammonia oxidation efficiency (Figure 4-8). The specific ammonia uptake rate was detected to decrease with the increase in influent LVX concentration, thus ammonia accumulation occurred. After some adaptation during Stage I, NH_4-N in R3 and R4 recovered. From Figure 4-5, the increase in effluent NO_2-N concentration signaled the recovery of nitrification in R3 and R4. The accumulation of NO_2-N might be associated with the inhibition both on denitrification and nitratation (from NO_2-N to NO_3-N) processes. Correspondingly, it was also noticed that the exposure of LVX exerted the notable effect on the effluent NO_3-N concentration (Figure 4-6). Yi et al. (2017) claimed that the activity of nitrite reductase could be inhibited by the presence of ciprofloxacin, one kind of new quinolones as same as LVX. During the exposure test, effluent TN concentrations increased, especially from R4. This observation might not only be attributable to inhibited denitrification, but also the

addition of LVX ($C_{18}H_{20}N_3O_4F$) which contains 11.7% of N (w/w). It has been reported before that the NO_3-N concentration obtained with the addition of antibiotic depended upon the balance between nitrification inhibition (which reduces the final concentration) and cell lysis (which increases the nitrogen pool in terms of ammonia and organic nitrogen, thus increasing the final concentration) (Alighardashi et al., 2009).

After stopping LVX exposure during Stage II, both R3 and R4 recovered, and after around 25 days' operation, all the reactors showed stably excellent performance again. During Stage III, all the 4 SBRs exhibited excellent stable performance in NH_4-N and PO_4-P removals. Although the LVX concentrations used in this study might be higher than those commonly encountered in WWTPs, the observed phenomena could help to shed light on the mechanisms responding to a sudden increase of antibiotic concentration due to the unexpected discharge of hospital effluents, or to increasing LVX use to control an epidemic. If an acute LVX exposure (lower than 128 mg-LVX/L like in this study) occurs, the microbial community in the sludge was changed leading to recovery the performance well, which might be the reason why most WWTPs are working well till now even though lots of antibiotic residues might already exist in the wastewater.

Therefore, Stage IV was initialed by re-exposing the reactors to LVX. It can be seen that NH_4-N was still easily and effectively converted in R1 and R4 (Figure 4-4). While the NH_4-N removals in other 2 reactors were affected to a great extent, due to re-exposure to 128 mg-LVX/L. This observation was probably brought about by the inhibition of AOB in R2 and R3 due to their high influent LVX concentrations, even though the bacteria already had LVX exposure experience (Figure 4-8). On the one hand, the effluent nitrite concentrations from R2 and R3 increased with the re-exposure of LVX in comparison to those from R1 and R4. Thus, in this study, the accumulation of NO_2-N in R2 and R3 might be associated with the inhibition both on denitrification and nitratation processes. Much lower NO_3-N concentrations were detected in R2 and R3 than those in R1 or R4 during the LVX re-exposure (Figure 4-6), in accordance with the exposure results. The effluent of TN concentrations from R2 and R3 were detected to increase obviously during the re-exposure period (Figure 4-7). In addition, better denitrification was achieved in R1 and R4 compared to R2 and R3 during the re-exposure stage (Stage IV). As reported previously, some antibiotics could inhibit important microbial processes like denitrification (Costanzo et al., 2005; Yi et al., 2017). Overall, the NO_2-N accumulation as well as lower NO_3-N production were observed during the re-exposure to the higher concentration of LVX.

After stopping the re-exposure of LVX (Stage V), the NH_4-N oxidation efficiencies of R2 and R3 could be recovered a lot. Thus, the AOB could function well again under no LVX conditions although it was much slower recovery than the first LVX exposure. While, the NO_2-N and NO_3-N concentrations in both R2 and R3 could not recover as the beginning level probably because nitrite-oxidizing bacteria (NOB) and denitrifying bacteria were small population (Figure 4-5 and Figure 4-6). It could be found that, after stopping re-exposure operation, AOB has recovered largely, while the nitratation processes are still inhibited in R2 and R3. In this study, it was found that re-exposure to 128 mg-LVX/L exerts more negative effects on the performance of R3 than R2. So, the detailed investigation focusing on microbial community analysis to reveal adaptation mechanism to LVX was performed in Chapter 5.

4.4. Summary

(1) LVX could inhibit the uptake and degradation of organics due to antibiotic functions while SBRs could recover organics removal efficiency quickly. Better DOC removal was achieved in R2 (IV) than R3 (IV) than R4 (I).

(2) The effluent LVX concentrations levels during re-exposure were similar with those during Stage I.

(3) NH_4-N removals were remarkably affected, most probably the inhibition of AOB by the high concentration of LVX, resulted in much lower nitritation efficiency. Ammonia oxidation ability recovered much slower during re-exposure.

(4) Overall, the NO_2-N accumulation as well as lower NO_3-N production were observed during the exposure/re-exposure to the higher concentration of LVX.

(4) During Stage V, NO_2-N concentration in both R2 and R3 could not recover as the beginning level and the nitratation processes are still inhibited in R2 and R3.

(5) TN increased, might not only be attributable to inhibited denitrification, but also the addition of LVX ($C_{18}H_{20}N_3O_4F$) which contains 11.7% of N (w/w). Re-exposure to 128 mg-LVX/L exerts more negative effects on the TN level of R3 than R2.

Table 4-1 Levofloxacin (LVX) concentrations (mg/L) in synthetic wastewater.

Reactor	Stage I	Stage II	Stage III	Stage IV	Stage V
R1	0	0	0	0	0
R2	4	0	0	128	0
R3	16	0	0	128	0
R4	128	0	0	4	0

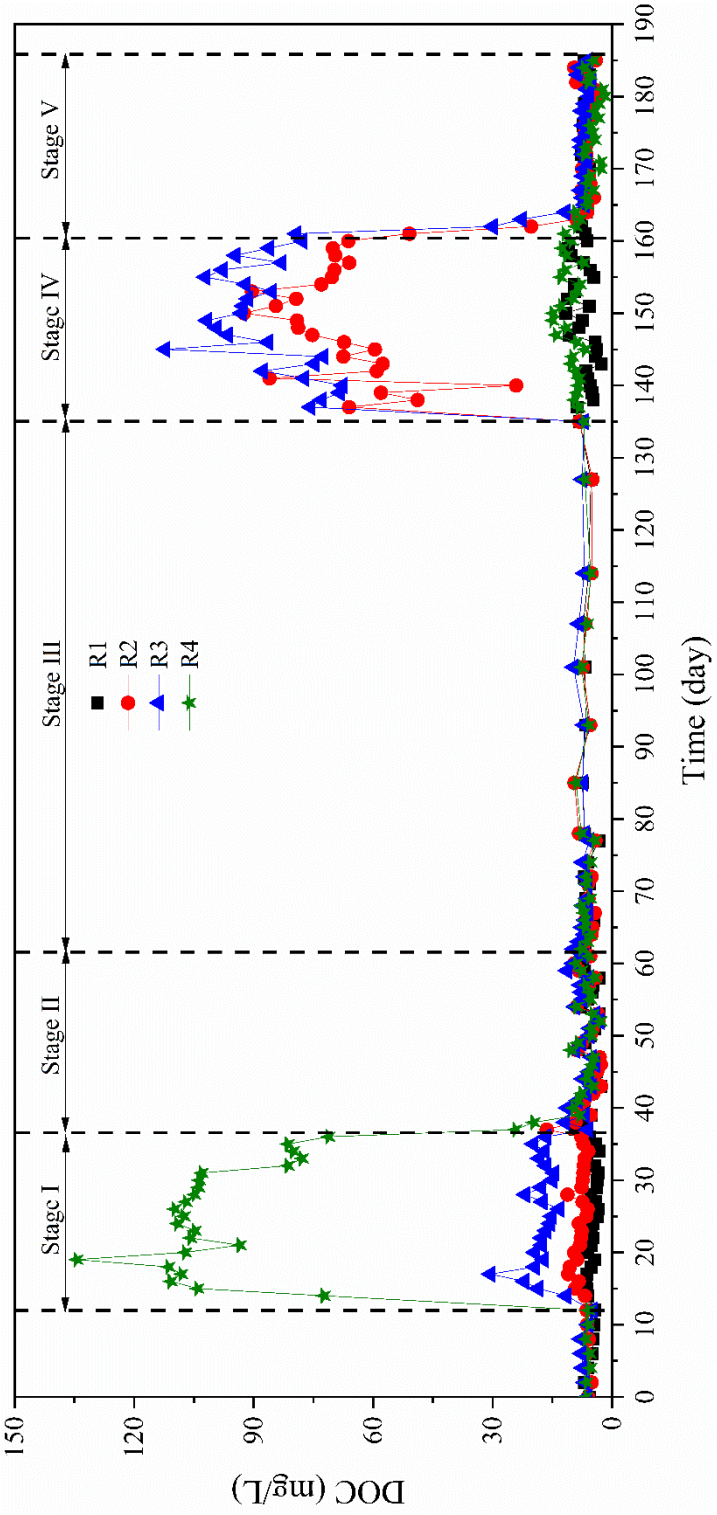

Figure 4-1 DOC concentration changes during fluctuated and staged LVX exposure. Stage I: R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L); Stage IV: R1 (0 mg-LVX/L); R2 (128 mg-LVX/L); R3 (128 mg-LVX/L); R4 (4 mg-LVX/L).

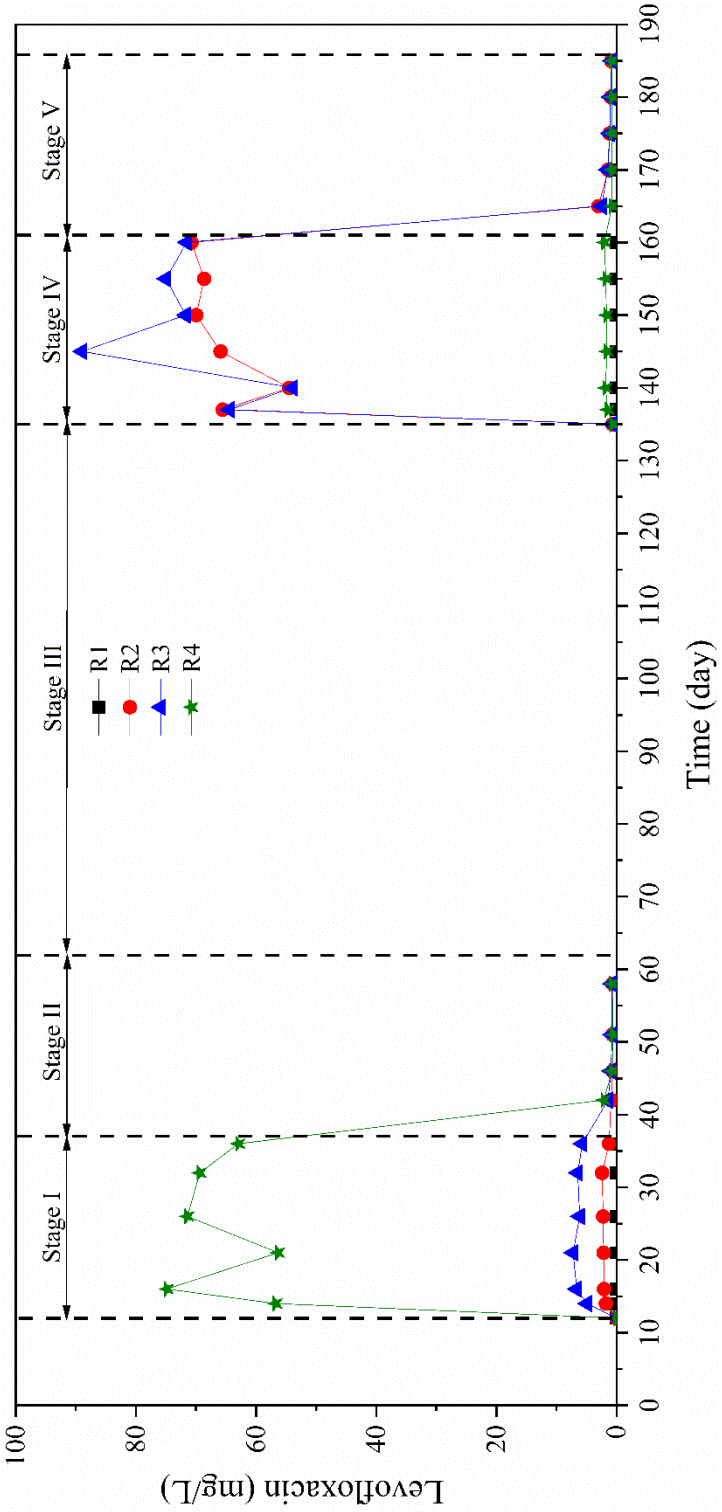

Figure 4-2 LVX concentration changes during fluctuated and staged LVX exposure. Stage I: R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L); Stage IV: R1 (0 mg-LVX/L); R2 (128 mg-LVX/L); R3 (128 mg-LVX/L); R4 (4 mg-LVX/L).

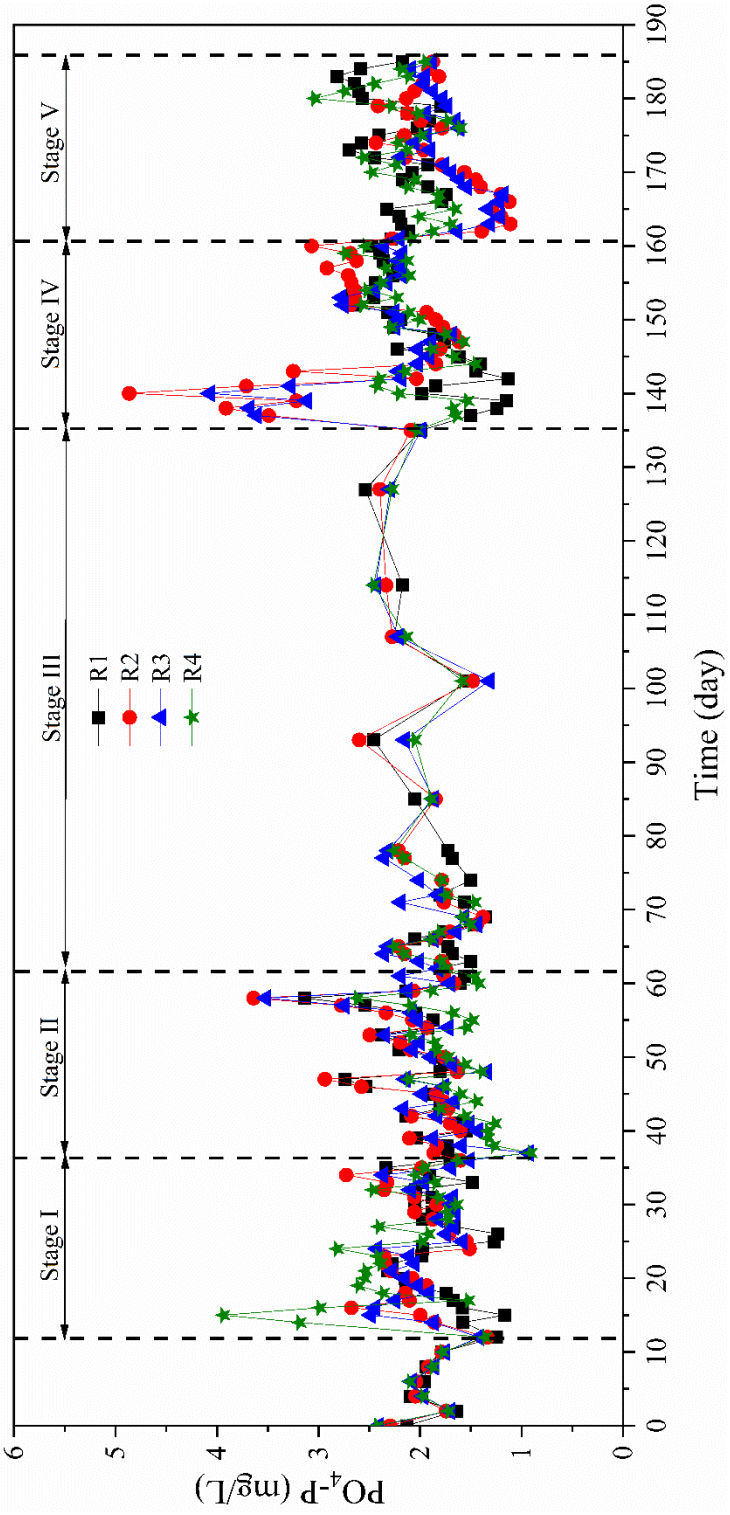

Figure 4-3 PO_4-P concentration changes during fluctuated and staged LVX exposure. Stage I: R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L); Stage IV: R1 (0 mg-LVX/L); R2 (128 mg-LVX/L); R3 (128 mg-LVX/L); R4 (4 mg-LVX/L).

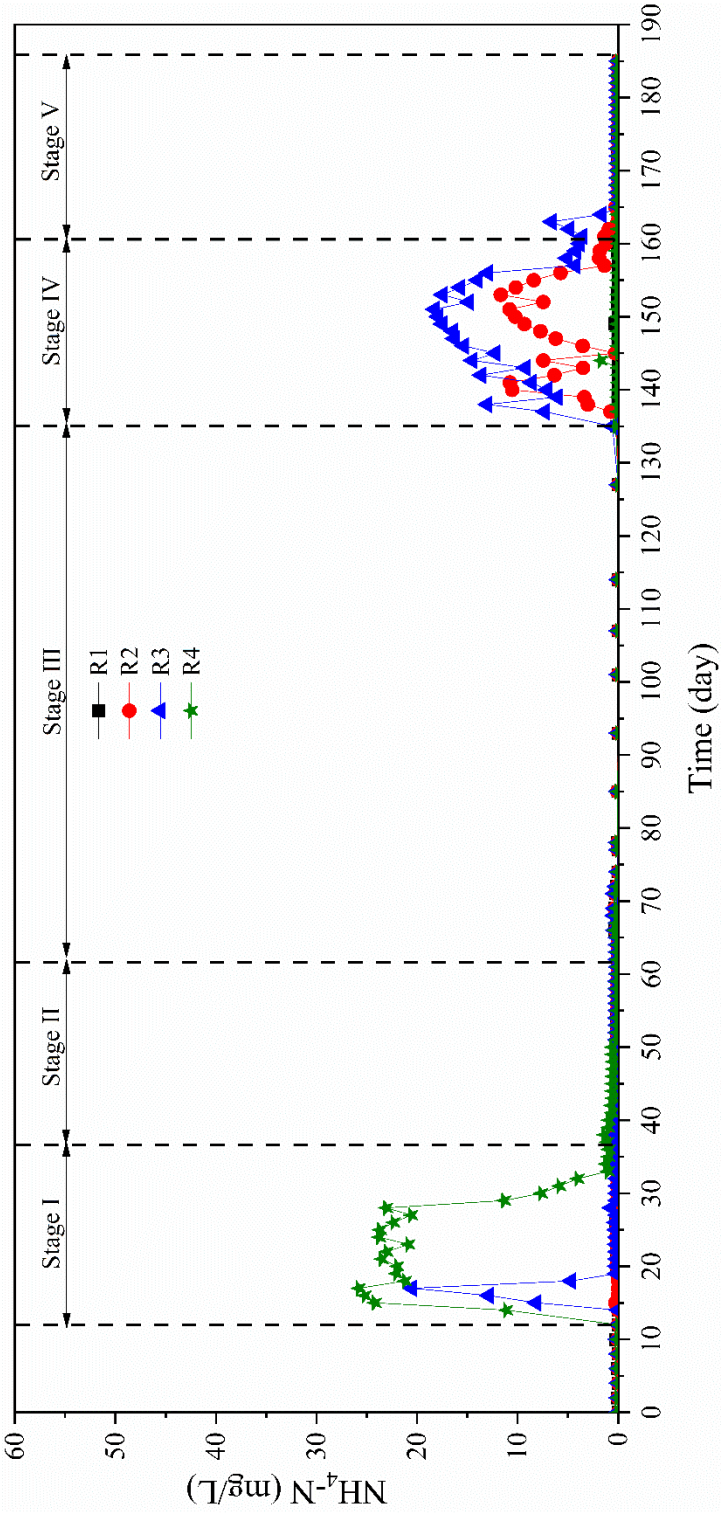

Figure 4-4 NH_4-N concentration changes during fluctuated and staged LVX exposure. Stage I: R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L); Stage IV: R1 (0 mg-LVX/L); R2 (128 mg-LVX/L); R3 (128 mg-LVX/L); R4 (4 mg-LVX/L).

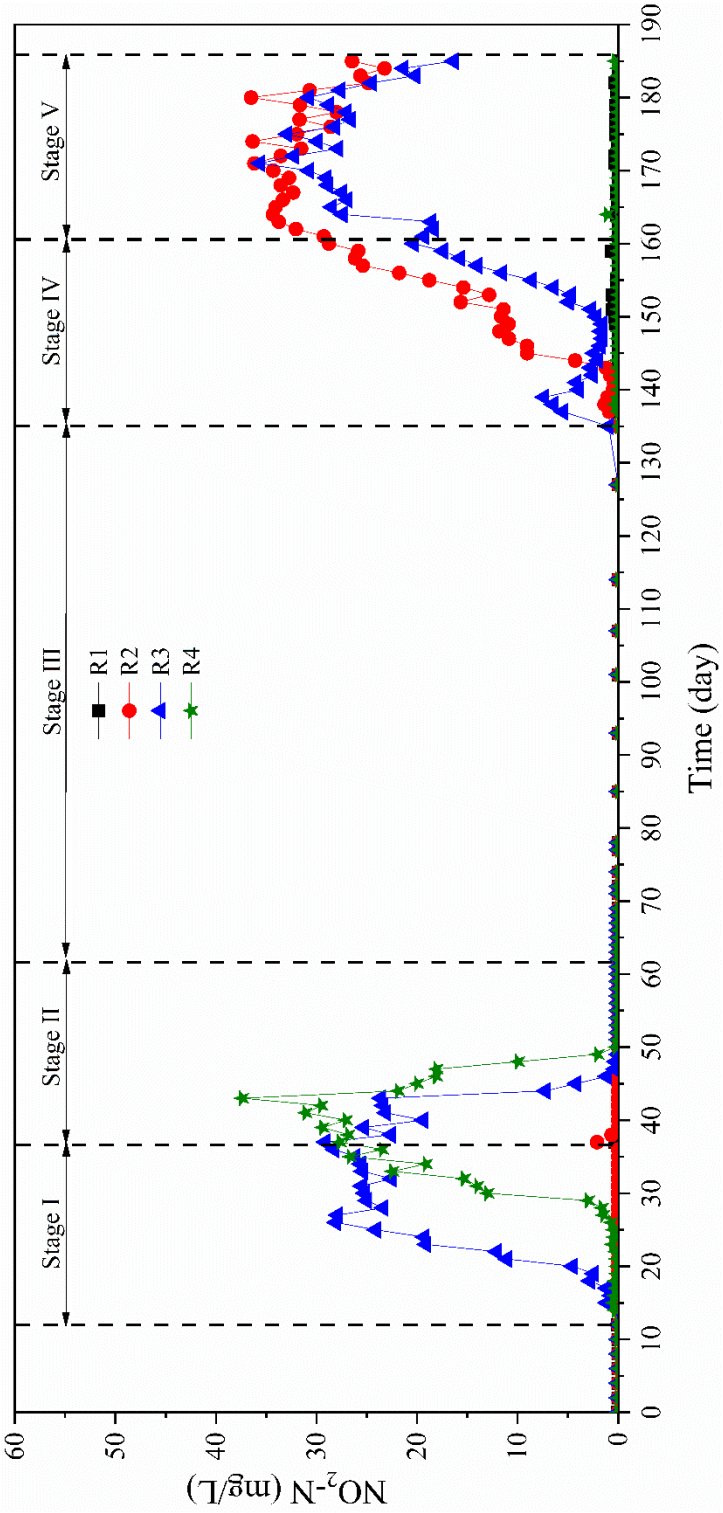

Figure 4-5 NO_2-N concentration changes during fluctuated and staged LVX exposure. Stage I: R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L); Stage IV: R1 (0 mg-LVX/L); R2 (128 mg-LVX/L); R3 (128 mg-LVX/L); R4 (4 mg-LVX/L).

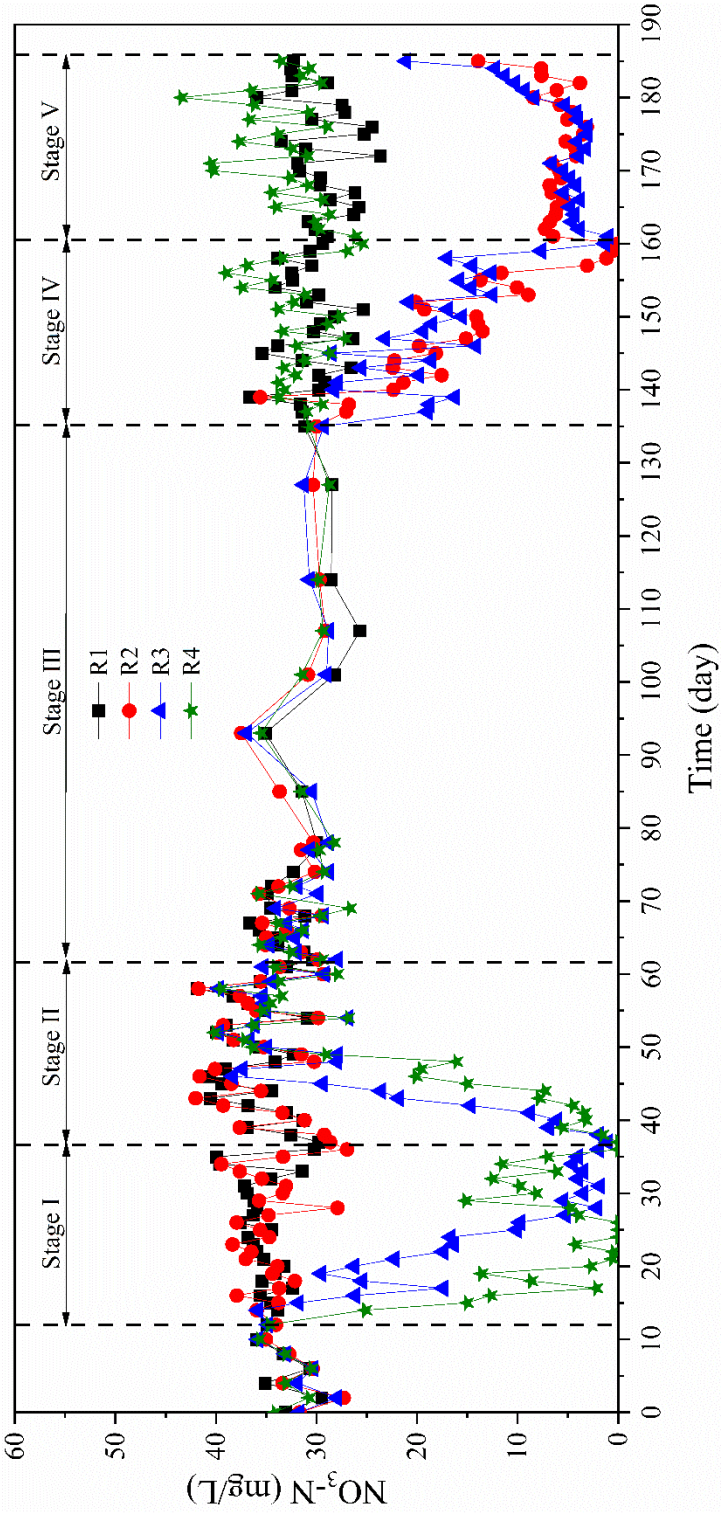

Figure 4-6 NO_3-N concentration changes during fluctuated and staged LVX exposure. Stage I: R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L); Stage IV: R1 (0 mg-LVX/L); R2 (128 mg-LVX/L); R3 (128 mg-LVX/L); R4 (4 mg-LVX/L).

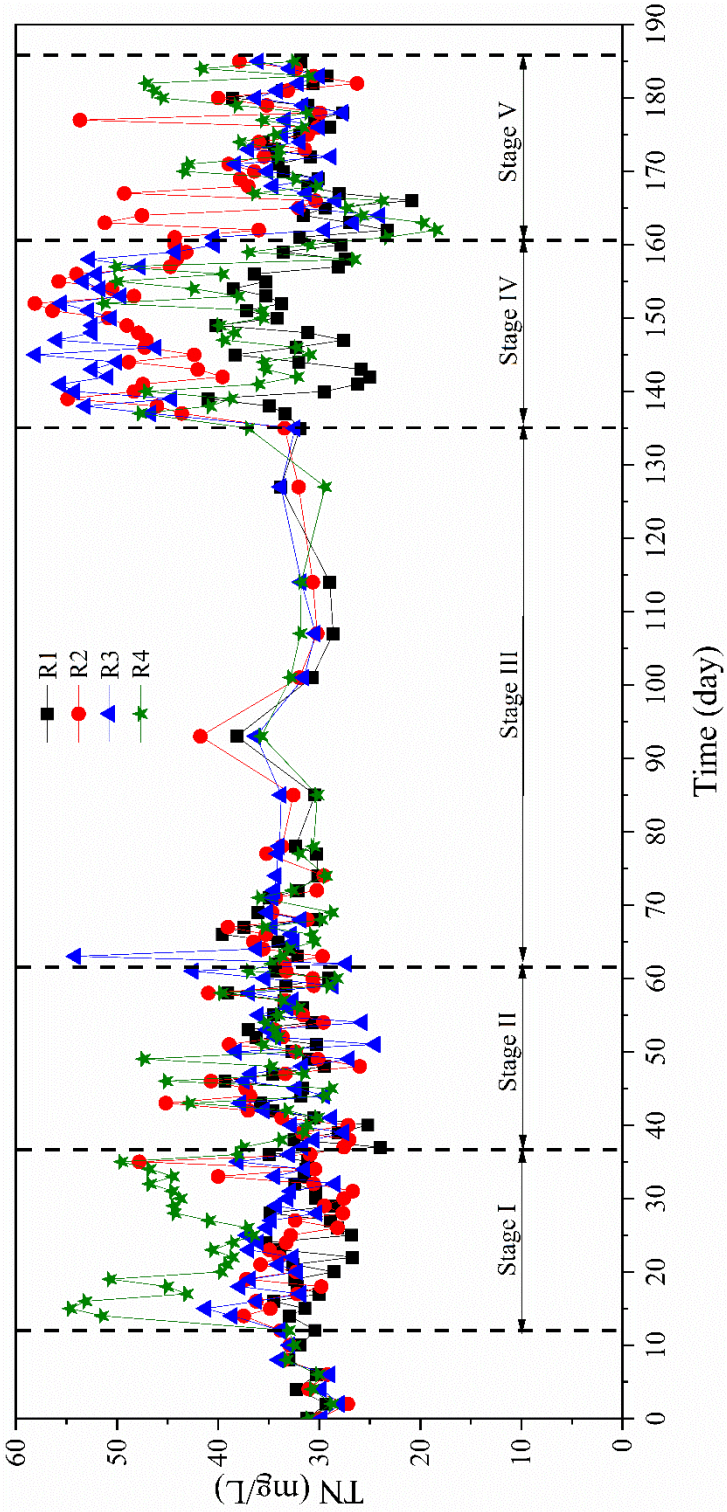

Figure 4-7 TN concentration changes during fluctuated and staged LVX exposure. Stage I: R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L); Stage IV: R1 (0 mg-LVX/L); R2 (128 mg-LVX/L); R3 (128 mg-LVX/L); R4 (4 mg-LVX/L).

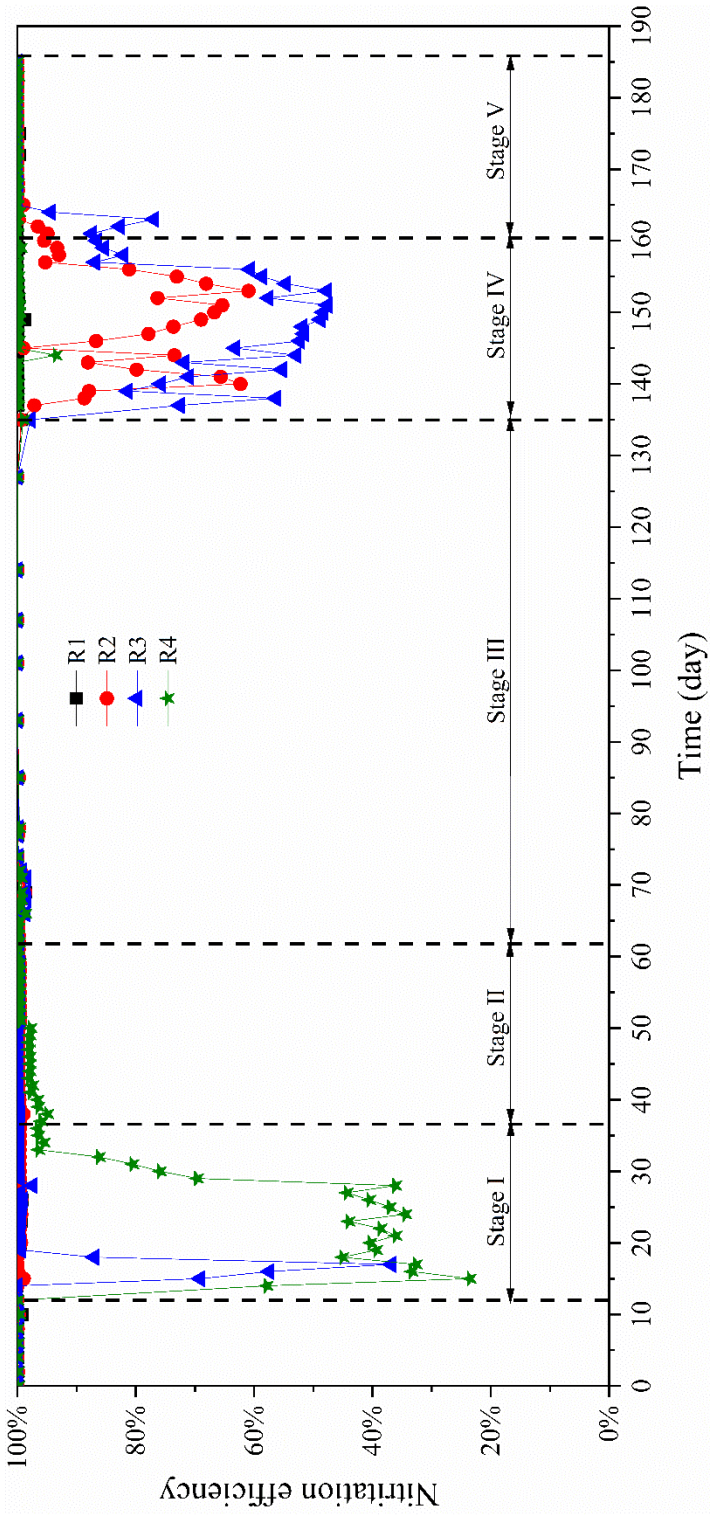

Figure 4-8 Nitritation efficiency changes during fluctuated and staged LVX exposure. Stage I: R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L); Stage IV: R1 (0 mg-LVX/L); R2 (128 mg-LVX/L); R3 (128 mg-LVX/L); R4 (4 mg-LVX/L).

Chapter 5 Dynamic Changes in Microbial Community Exposed to LVX

5.1. Introduction

The water environment has a direct contact with human life due to the wide usage of water, while the continuity of the water may transport antibiotic/resistance to various places around the world, which could pose threat to public health and ecosystems (Ding and He, 2010). In some studies, such as the nitrogen conversion process, changes in the ecological function of the aquatic environment caused by antibiotics have been studied. 350 ng/L ciprofloxacin has reported to lead to AOB reduction in a laboratory-scale underwater biofilter (Gonzalez-Martinez et al., 2014). However, sometimes, complex bacterial mixtures exposed to antibiotics could increase the nitrification activity, however, with no clear reasons (Halling-Sørensen, 2001). Another interesting study of SBR showed that continuous exposure to trace erythromycin (100 μg / L) did not significantly change nitrogen or phosphorus removal, but microbial community structures shifted significantly (Fan et al., 2009). This means that functional redundancy within the SBR could compensate for the loss of sensitive bacteria with antibiotics exposures.

The research focusing on antibiotics contamination already have reported many, but most of antibiotics are made by microorganisms. That is why the antibiotics from human activity are hard to analyze. So, we focus on quinolones that is completely synthetic antibiotics. No degrader and producer of quinolones live in environment as so far. The microbial community structure of the WWTPs is likely to be affected by the exposure of antibiotics. To much more deeply explore the mechanisms of bacteria committee with the presence of fluctuated and staged LVX during wastewater treatment in the SBRs, it is necessary to investigate the microbial community structure shift to shed light on microbial responses to LVX. This chapter investigated the adaptation mechanisms of bacteria community exposed to acute and chronic/fluctuated LVX levels during wastewater treatment in SBRs. This study also illustrated the importance of the control of residual antibiotics concentration not to be ARB sources and showed the resilience on residual antibiotics exposure by the adaptation of bacterial community to residual antibiotics contamination for nitrogen removal.

5.2. Materials and methods

5.2.1. Microbial community analysis

The sludge samples were obtained taken from the four SBRs during various stages (day

10 before Stage I, days 17, 24 and 32 during Stage I, days 42, 47, 52 and 57 during Stage II, day 133 during Stage III, days 140, 145, 150, 155 and 160 in Stage IV, and days 165, 170, 175, 180 and day185 during Stage V). Total DNA was extracted using ISOIL for Beads Beading Kit (Nippon Gene, Japan) according to the manufacturer's protocol. The amplicon library of 16S rRNA gene sequence was made by 2-step PCR according to a previous research (Miya et al., 2015) in which SimpliAmp Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, USA) was applied.

The first PCR was carried out by 515F-806R primer set (5′-GTG CCA GCM GCC GCG GTA A-3′ and 5′-GGA CTA CHV GGG TWT CTA AT-3′) targeted to the 16S rRNA gene for the second PCR (Caporaso et al., 2011). The polymerase was used Ex Taq (Takara Bio Inc., Shiga, Japan) with 10 ng of template DNAs according to the manufacturer's protocol. The thermal cycle reaction was carried out in 25 cycles: 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. The PCR products were purified using AMpure XP (Beckman Coulter, Inc., Brea, USA) and then the purified PCR products were used as the templates for the second PCR.

The second PCR was tailed PCR for addition to index sequence and adapter sequence for next generation sequencer using MiSeq (Illumina, Inc., San Diego, USA). The polymerase was used Ex Taq (Takara Bio Inc., Shiga, Japan) with 6 μL of purified the first PCR amplified as templates. The thermal cycle reaction was carried out in 8 cycles: 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. The second PCR products were purified using AMpure XP (Beckman Coulter, Inc., Brea, USA) and then assayed with a Qubit (Thermo Fisher Scientific Inc., Waltham, USA) for DNA concentration determination.

All samples were adjusted to isodensity with Buffer EB and then all samples were mixed to make a sequence sample. After that, the mixed sample was re-purified using BluePippin (Sage Science, Inc., Beverly, USA) to prepare the library for amplicon analysis. The amplicon library was sequenced using a MiSeq (Illumina, Inc., San Diego, USA) at the Biotechnology Center of Akita Prefectural University. All the sequence data were classified phylogenetically using Claident v0.2 (https://www.claident.org). The quality-filtering sequences were divided into unique operational taxonomic units (OTUs) at the 97% level.

5.2.2. Statistical analysis methods

Non-metric multidimensional scaling (NMDS) and calculation of Simpson index was performed based on the rRNA gene quantification results using the package 'vegan' (Oksanen et al., 2009) for R (R Development Core Team, 2008). A quantitative microbial community profile can be shown as one data point on an NMDS plot, and thus changes in microbial

community structure can be visualized by connecting consecutive data points. Highly similar sets are plotted close together. Simpson's index is a measure of diversity. In this study, the value ranges between 0 and 1, which the greater value shows the greater the sample diversity.

5.3. Results and discussion

5.3.1. Richness and diversity

To analyze the effect of LVX exposure on microbial diversity, high-throughput sequencing analysis was conducted, and then Simpson index and NMDS were analyzed. High-quality reads were obtained for the samples taken from the four SBRs during various stages (day 10 before Stage I, days 17, 24 and 32 during Stage I, days 42, 47, 52 and 57 during Stage II, day 133 during Stage III, days 140, 145, 150, 155 and 160 in Stage IV, and days 165, 170, 175, 180 and 185 during Stage V). The Figure 5-3, Figure 5-4, Figure 5-5 and Figure 5-6 have shown the relative bacterial community abundances at the phylum level, but only proteobacteria show class level such as alpha-proteobacteria, and so on. The results indicate that the microbial community in the reactors during the high concentrations of LVX exposure exhibits a lower diversity (Figure 5-3, Figure 5-4, Figure 5-5, Figure 5-6 and Figure 5-1). The sharply decrease in Simpson index reflected the bacterial diversity in the SBRs significantly decreased with the LVX compared with R1 (Figure 5-1). After stop of first time LVX exposure (Stage II), the values of Simpson index have increasing trend in R2, R3 and R4, indicating recovery of the microbial diversity. Actually, several phylums were increased, which were Chloroflexi, Cyanobacteria, Planctomycetes, and delta-proteobacteria in R2, and Acidbacteria, Armatimonadetes, Chlamidiae, Cyanobacteria, Nitrospiparae, Planctomycetes, beta-proteobacteria, delta-proteobacteria, and gamma-proteobateria in R3, and Acidbacteria, Chlamydiae, Chloroflexi, Cyanobacteria, Firmicutes, Nitrospirae, Verrucomicrobia, delta-proteobacteria, and gamma-proteobacteria in R4 (Figure 5-3, Figure 5-4, Figure 5-5 and Figure 5-6). While during the Stage V, the Simpson index of samples from R2 increased much obviously than R3, reflecting that the bacterial diversity in R2 recovered much better and faster than R3 although with the same re-exposure concentrations (128 mg-LVX/L). Since LVX has toxicity on microorganism and reduced the microbial population due to not only inhibition of DNA gyrase but also production of radicals to kill the bacteria (Drlica and Zhao, 1997; Kohanski et al., 2007; Yi et al., 2017), the diversity was decrease on exposure duration and was increase on no-exposure duration.

Meanwhile, NMDS analysis indicate changes in bacterial community structure because of avoiding the assumption of linear relationships among variables. Also, NMDS is reported to be

one of the most generally effective ordination method for ecological community data (McCune and Grace, 2002). The NMDS plot from the analysis of sludge samples demonstrated the continuous shifts in community structure (Figure 5-2). Each point stands for one sludge sample. The bacterial communities in sludge samples before Stage I gathered in and around quadrant 4 of the NMDS plot as the figure shown (Figure 5-2), but they shifted widely and totally differently with the progress of treatment. The community profiles from R1 were located near with each other, indicating that almost no significant change in community structure. The community profiles from R4 were widely dispersed and distantly located from the profiles from the other SRBs, particularly within the Stage I period (exposure to 128 mg-LVX/L), but it seems much faster to adapt in R4 during the Stage IV (re-exposure to 4 mg-LVX/L). A relatively more significant change in community structure between two consecutive time points on the plot was relevant to the adaptation with the different exposure of various LVX concentrations, also reflected by R2 and R3 in Figure 5-2. These suggested that the dynamic changes in bacterial community structures were well reflected in the NMDS results, indicating that the different concentrations LVX exposure was a crucial factor to control the quantitative evolution of communities in our systems as showing the arrows in Figure 5-2.

5.3.2. Identification of the involved microbes for nitrogen removal restoration

To identify the involved microbes for nitrogen removal restoration in the SBRs, the involved bacterial abundance analysis was conducted. The most obvious difference between community samples with or without LVX exposure was the distribution of phylum Proteobacteria in the total community composition (Figure 5-3, Figure 5-4, Figure 5-5 and Figure 5-6). This phenomenon is possibly due to the fact that it was the known phylum related to nitrogen removal (Yi et al., 2017; You et al., 2009). It can be seen that at the phylum level, Proteobacteria was always the predominant in the control. However, with the increase of the LVX concentration, the proportion of Proteobacteria shifts dramatically. It was reported that a lot of denitrifier belonged to Proteobacteria (Yi et al., 2017). It has been reported before that AOB commonly belong to the Beta- and Gamma-proteobacteria, including *Nitrosomonas* sp. (Beta-proteobacteria), *Nitrosospira* sp. (Beta-proteobacteria), and *Nitrosococcus* sp. (Gamma-proteobacteria) (You et al., 2009). *Nitrosomonas*/*Nitrosospira* species appear to dominate the natural and engineering systems, therefore, AOB of the Beta-proteobacteria have been used as model organisms in microbial ecological studies (Kowalchuk and Stephen, 2001; Nold et al., 2000; Park et al., 2002; Whitby et al., 1999). In order to get more related detail information, the phylum of proteobacteria has been subdivided into lots of classes as shown in Figure 5-3, Figure

5-4, Figure 5-5 and Figure 5-6. Lower relative abundance of Beta-proteobacteria was detected in the samples exposed to as high as 128 mg-LVX/L, while much higher was detected with the adaptation in the SBRs. Bacteria belongs to Gamma-proteobacteria seems more sensitive than those belonging to Alpha-proteobacteria (Figure 5-3, Figure 5-4, Figure 5-5 and Figure 5-6). Meanwhile, Bacteroidetes showed a different increase trend at the beginning of the exposure, implying a selective enrichment, whereas Bacteroidetes increased after exposure to LVX immediately, especially when the community was exposed 128 mg-LVX/L. While with the adaptation of bacterial communication in samples due to phase change, the relative abundance of Bacteroidetes decreased gradually. This phenomenon is possibly due to the fact that Proteobacteria might be more sensitive to residue new quinolones such as LVX than Bacteroidetes. Compared with the control samples, the percentages of Nitrospirae was found to change a lot in the reactors, indicating their sensitivity to LVX.

High-throughput sequencing analysis is an effective means for better and deeper understanding of the microbial community structure in various environmental processes. The percentages of nitrifying bacteria in bacterial communities in the samples was illustrated in Figure 5-7, Figure 5-8, Figure 5-9 and Figure 5-10. Standing on the genus level could further infer the functions of the involved communities. Some critical species that might be responsible for nitrogen removal restoration from LVX exposure were observed in the community because nitrogen removal is closely related to the abundances of some key microorganisms. Generally, there are main types of AOB, i.e., *Nitrosomonas* sp. and *Nitrosospira* sp. present in WWTPs. In this work, *Nitrosomonas* sp. was detected as dominant in all the four reactors, and its abundance was affected significantly, but quite less *Nitrosospira* sp. was several sampling times. The results of *Nitrosomonas* sp. percentages against total bacterial population shown in Figure 5-7, Figure 5-8, Figure 5-9 and Figure 5-10 have reflected obvious trend with the recovery and tolerance during and after LVX exposure. At the beginning of LVX exposure, the relative abundance of *Nitrosomonas* sp. decreased immediately as shown from Stage I and Stage IV. In a partial-nitritation bench-scale submerged biofilter, it has been found that 350 ng/L of ciprofloxacin which is a new quinolone as well as LVX caused reduction of AOB (Gonzalez-Martinez et al., 2014), but exposure by 4 mg-LVX/L did not affect on the abundance of *Nitrosomonas* sp. (stage I in Figure 5-8 and stage IV in Figure 5-10), but after exposure the abundance of *Nitrosomonas* sp. quickly recover (stage II in Figure 5-9 and Figure 5-10, and stage V in Figure 5-8 and Figure 5-9). *Nitrosomonas* sp. has shown ability to tolerance LVX exposure when exposed to lower concentrations of LVX (4 mg-LVX/L). Comparison of

exposure by 128 mg-LVX/L on stage I in R4 and stage IV in R2 and R3, the abundance of *Nitrosomonas* sp. could not recover within the first exposure time in R4 on stage I, but that of *Nitrosomonas* sp. was observed in R2 and R3 on stage IV. Thus, the results could show adaptation ability such as tolerance/resistance got better during the re-exposure time. Although *Nitrospira* sp. and *Nitrobacter* sp. are widely known as the two major types of NOB in wastewater treatment systems (Daims et al., 2000), *Nitrobacter* sp. was merely measured in all the SBRs. It was found that the presence of LVX reduced the abundance of *Nitrospira* sp. Although *Candidatus Nitrotoga* sp. also detected in the samples before the exposure of LVX, it was very sensitive to the toxicity of LVX and the accounts were very low. While *Nitrospira* sp. were recovered much more slowly than *Nitrosomonas* sp. after the exposure, especially exposed to high concentrations of LVX. During re-exposure time, *Nitrospira* sp. showed much better tolerance to LVX compared to first time exposure, and the tolerance could be better than *Nitrosomonas* sp. (Figure 5-7, Figure 5-8, Figure 5-9 and Figure 5-10). Since the above results were in accordance with NH_4-N, NO_2-N, NO_3-N and TN profiles shown in Figure 4-4, Figure 4-5, Figure 4-6 and Figure 4-7, the key bacteria were *Nitrosomonas* sp. and *Nitrospira* sp. Thus, the bacteria could be used as the biomarker for nitrogen removal in treatment.

Around 11 types of denitrifying bacteria, such as *Thauera* sp., *Azoarcus* sp., *Hyphomicrobium* sp., *Rhodobacter* sp. were mainly detected in either the control or the LVX exposed SBRs as Figure 5-11, Figure 5-12, Figure 5-13 and Figure 5-14. Among them, *Thauera* sp. was the predominant denitrifier in all the four reactors. It was observed that LVX significantly reduced the abundance of this predominant denitrifier, *Thauera* sp., though other types of denitrifier were also affected at some extent. *Thauera* sp. often occurs in denitrifying conditions, nitrate, nitrite, and oxygen are used as electron acceptor (Schramm et al., 2000). However, the abundance of *Thauera* sp. was severely inhibited by LVX higher than 4 mg-LVX/L. During the Stage II, the abundance of *Thauera* sp. gradually recovered along with time. While it could be seen from Figure 5-11, Figure 5-12, Figure 5-13 and Figure 5-14 that *Azoarcus* sp. and *Flavobacterium* sp. showed much more adaptation ability during the staged operation time.

ARB can create crucial situation for chemotherapy worldwide. The bacteria from WWTPs have been considered as the source of ARB in the aquatic environment. In this study, *Acinetobacter* sp. belonging to gamma-proteobacteria, an important bacterium for multi-drug resistant increased the abundance during high concentration of LVX exposure, but during no exposure time, that decreased due to being beaten in a competence (Figure 5-15, Figure 5-16,

Figure 5-17 and Figure 5-18). The abundance of *Chryseobacterium* sp. belonging to Bacteroidetes, one of opportunistic infection was found to increase during high LVX concentration exposure. However, during the non-exposure period, this species was detected to decrease, possibly due to its lower competitiveness under the tested conditions. The results suggested the control of residual antibiotics concentration in WWTPs is important to control ARB in WWTPs. Consequently, when WWTPs have very low concentration of residual antibiotics, the WWTPs could not be ARB sources. This study illustrates the importance of the control of residual antibiotics concentration not to be ARB sources and showed the resilience on residual antibiotics exposure by the phase change of bacterial community for nitrogen removal.

5.4. Summary

(1) The sharply decrease in Simpson index reflected the bacterial diversity in the SBRs significantly decreased with the LVX compared with R1. Since LVX has toxicity on microorganism and reduced the microbial population due to not only inhibition of DNA gyrase but also production of radicals to kill the bacteria, the diversity was decrease on exposure duration and was increase on no-exposure duration.

(2) The NMDS plot from the analysis of sludge samples demonstrated continuous shifts in community structure. The results suggested that the dynamic changes in bacterial community structures were well reflected in the NMDS results, indicating that the different concentrations LVX exposure was a crucial factor to control the quantitative evolution of communities in our systems.

(3) The recovery of biological nutrients removal performance was retarded by LVX exposure, due to the fact that the key bacteria, i.e. *Nitrosomonas* sp. (AOB) and *Nitrospira* sp. (NOB) decreased, and that *Thauera* sp., the predominant denitrifiers were reduced during the LVX exposure period. However, after stopping exposure their population was quickly increased and thus the performance was recovered. The abundances of Proteobacteria phylum were always the predominant in the control, however, the proportion shifts dramatically with the exposure of LVX concentration. *Acinetobacter* sp. that important bacteria for multi-drug resistant on chemotherapy increased the abundance during high concentration of LVX exposure, but during no exposure time, that decreased due to being beaten in a competence.

(4) The results showed the control of residual antibiotics concentration in WWTPs is important to control ARB in WWTPs.

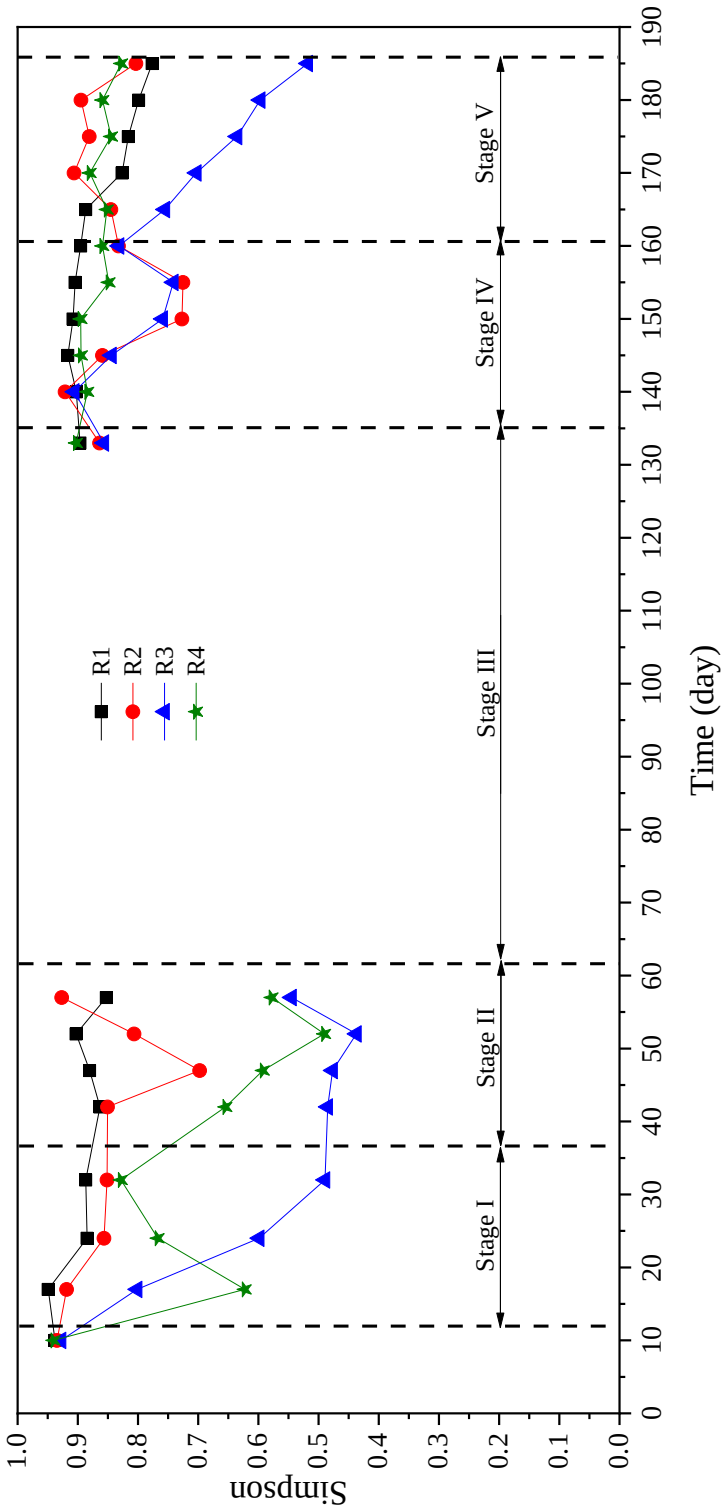

Figure 5-1 Simpson index of sludge samples taken from SBRs.

Stage I: R1 (0 mg-LVX/L); R2 (4 mg-LVX/L); R3 (16 mg-LVX/L); R4 (128 mg-LVX/L);

Stage IV: R1 (0 mg-LVX/L); R2 (128 mg-LVX/L); R3 (128 mg-LVX/L); R4 (4 mg-LVX/L);

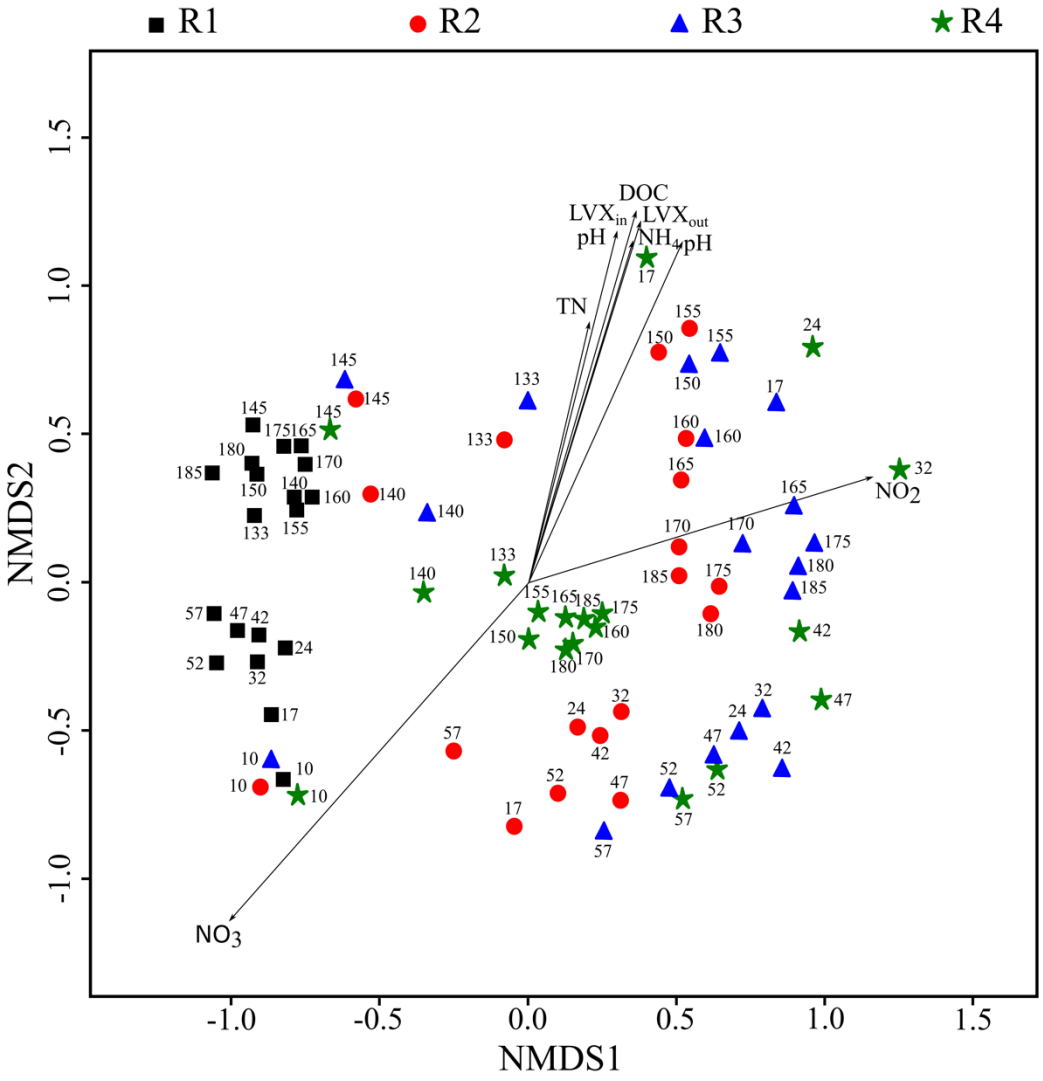

Figure 5-2 NMDS analysis of sludge samples taken from SBRs.

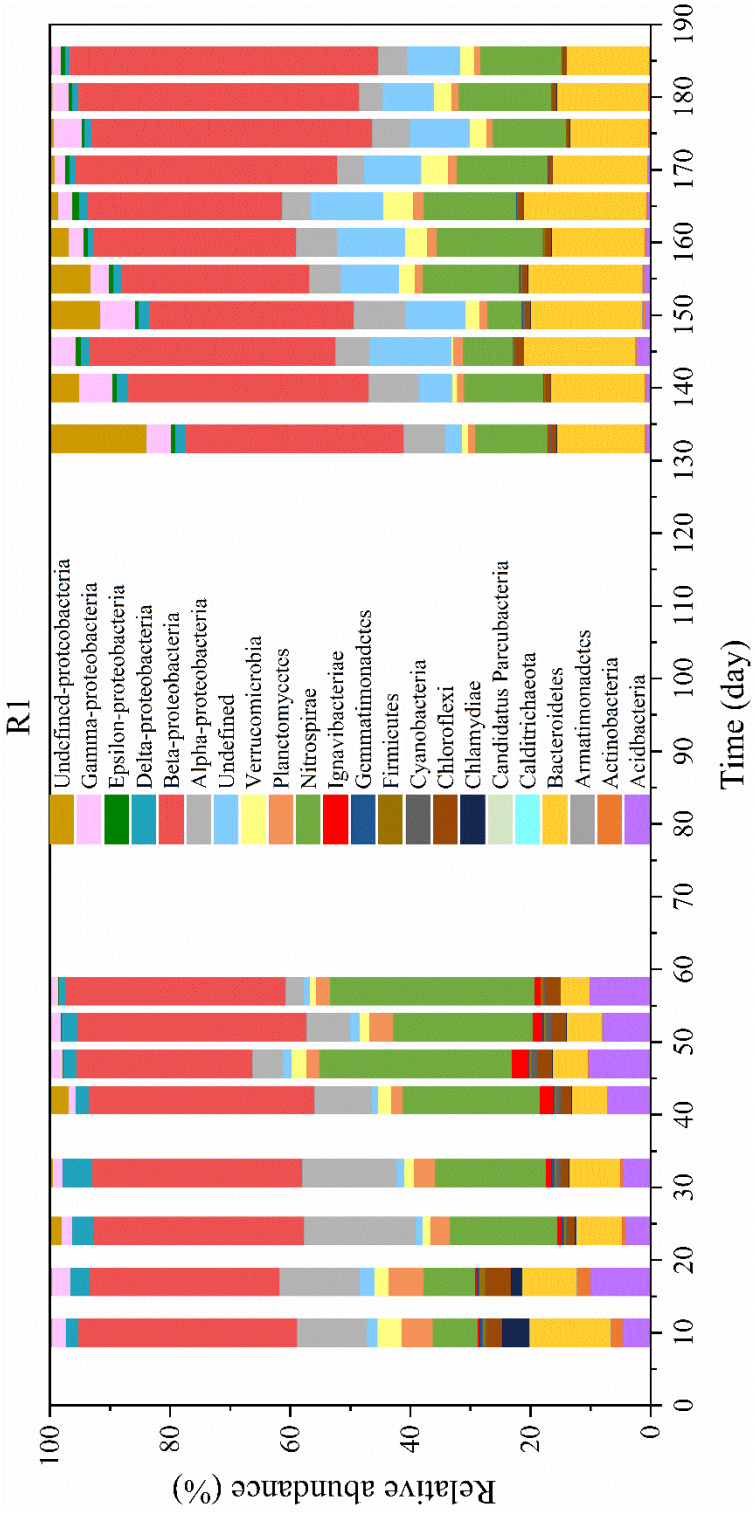

Figure 5-3 Phylum and class diversity of sludge samples taken from R1.

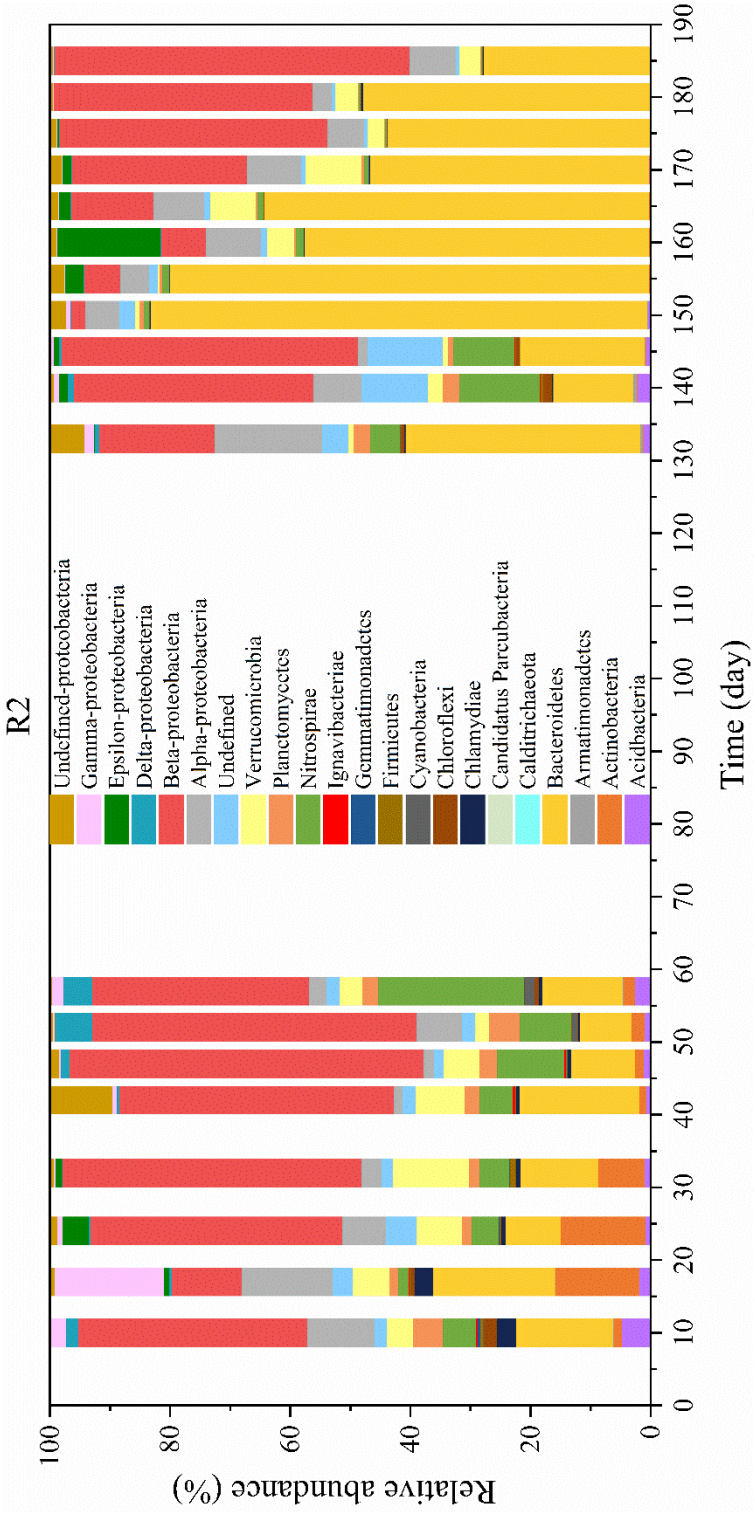

Figure 5-4 Phylum and class diversity of sludge samples taken from R2.

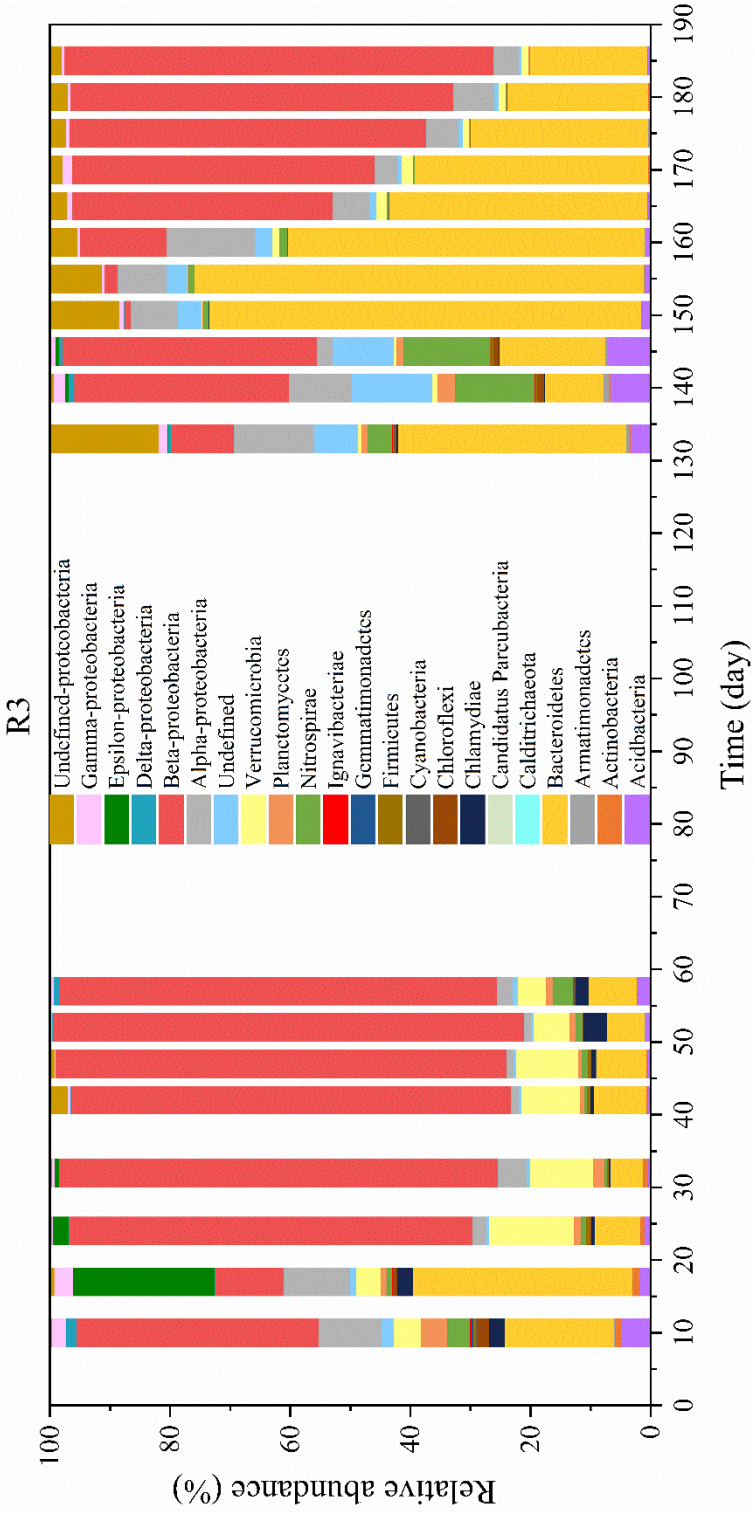

Figure 5-5 Phylum and class diversity of sludge samples taken from R3.

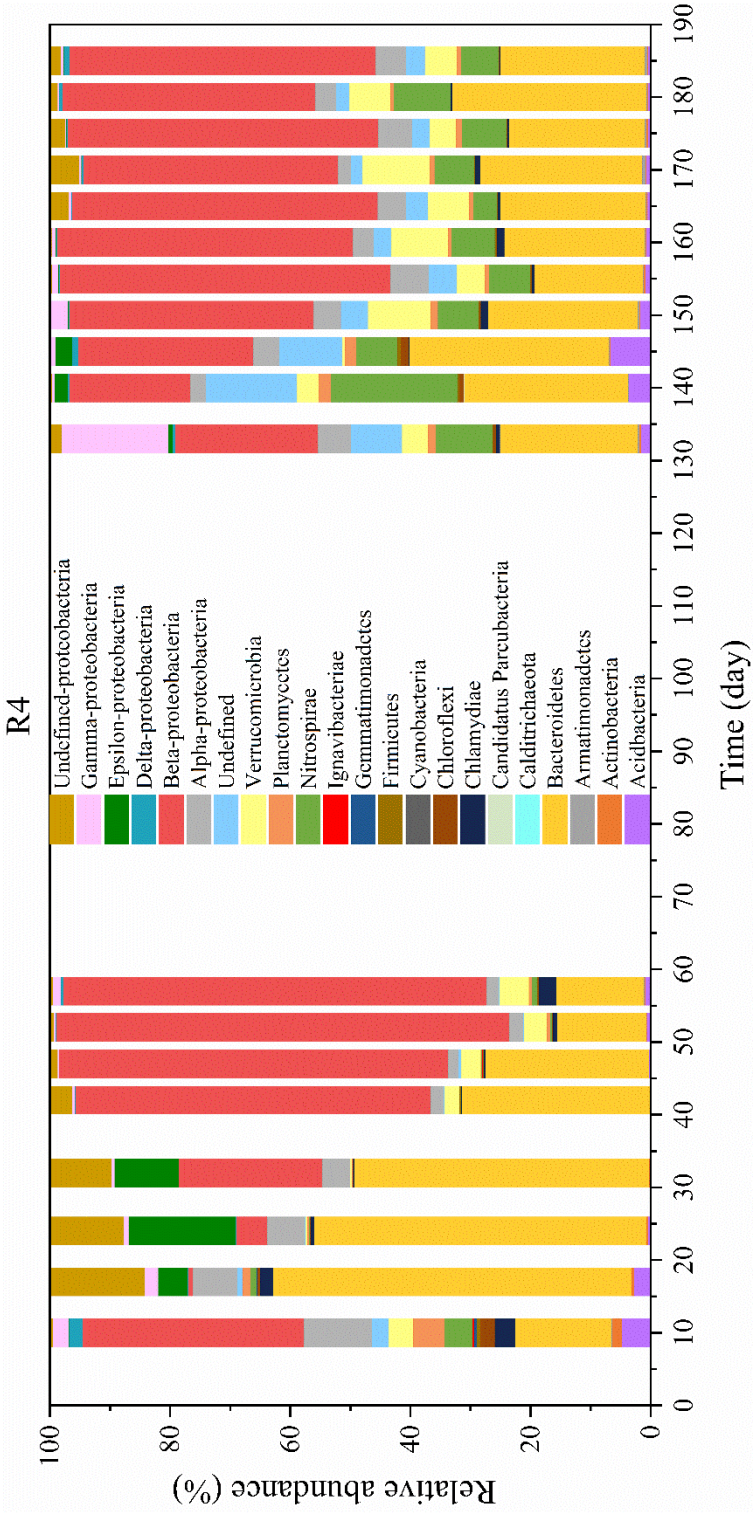

Figure 5-6 Phylum and class diversity of sludge samples taken from R4.

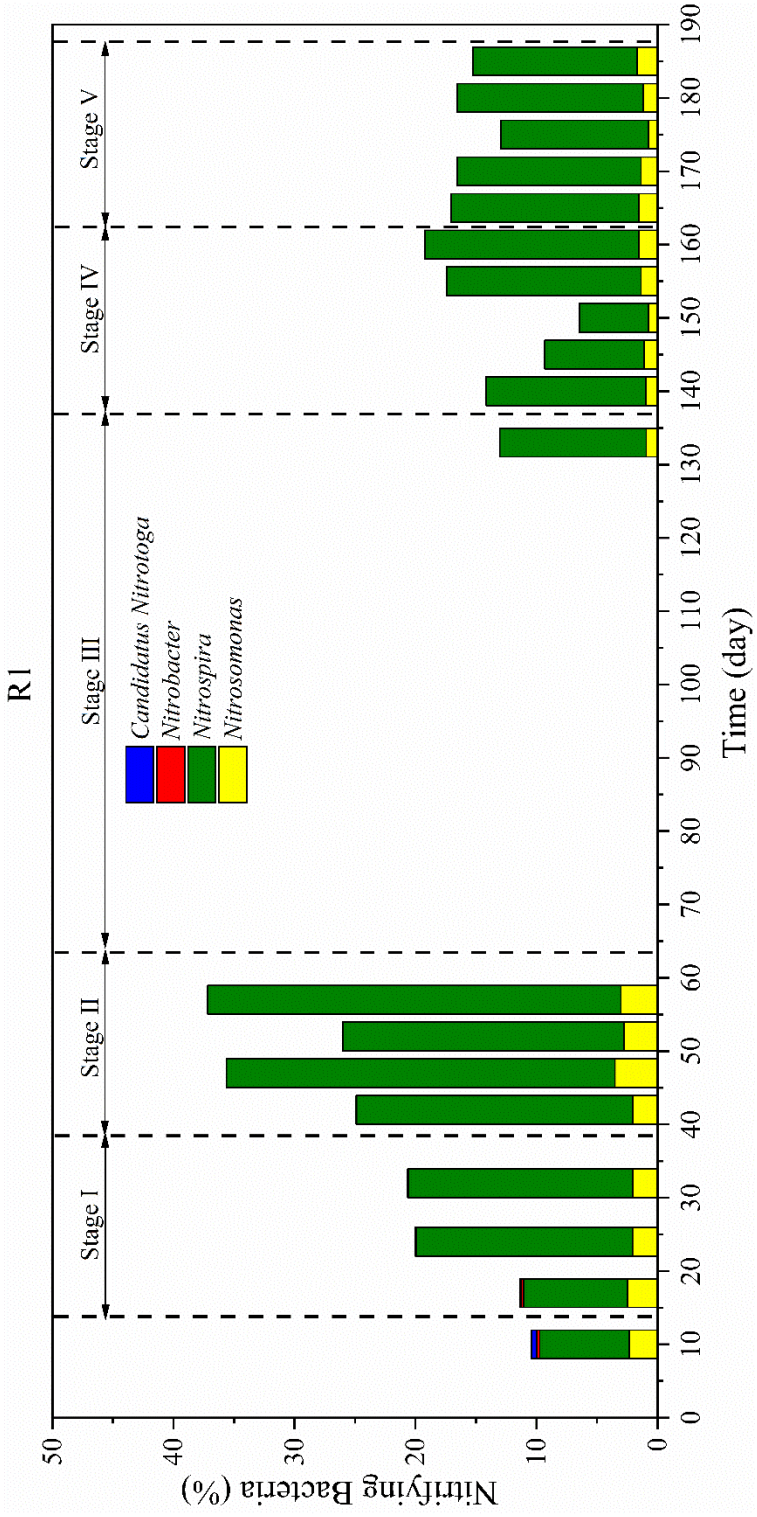

Figure 5-7 Percentage changes of nitrifying bacteria in sludge samples taken from R1. Stage I: R1 (0 mg-LVX/L); Stage IV: R1 (0 mg-LVX/L).

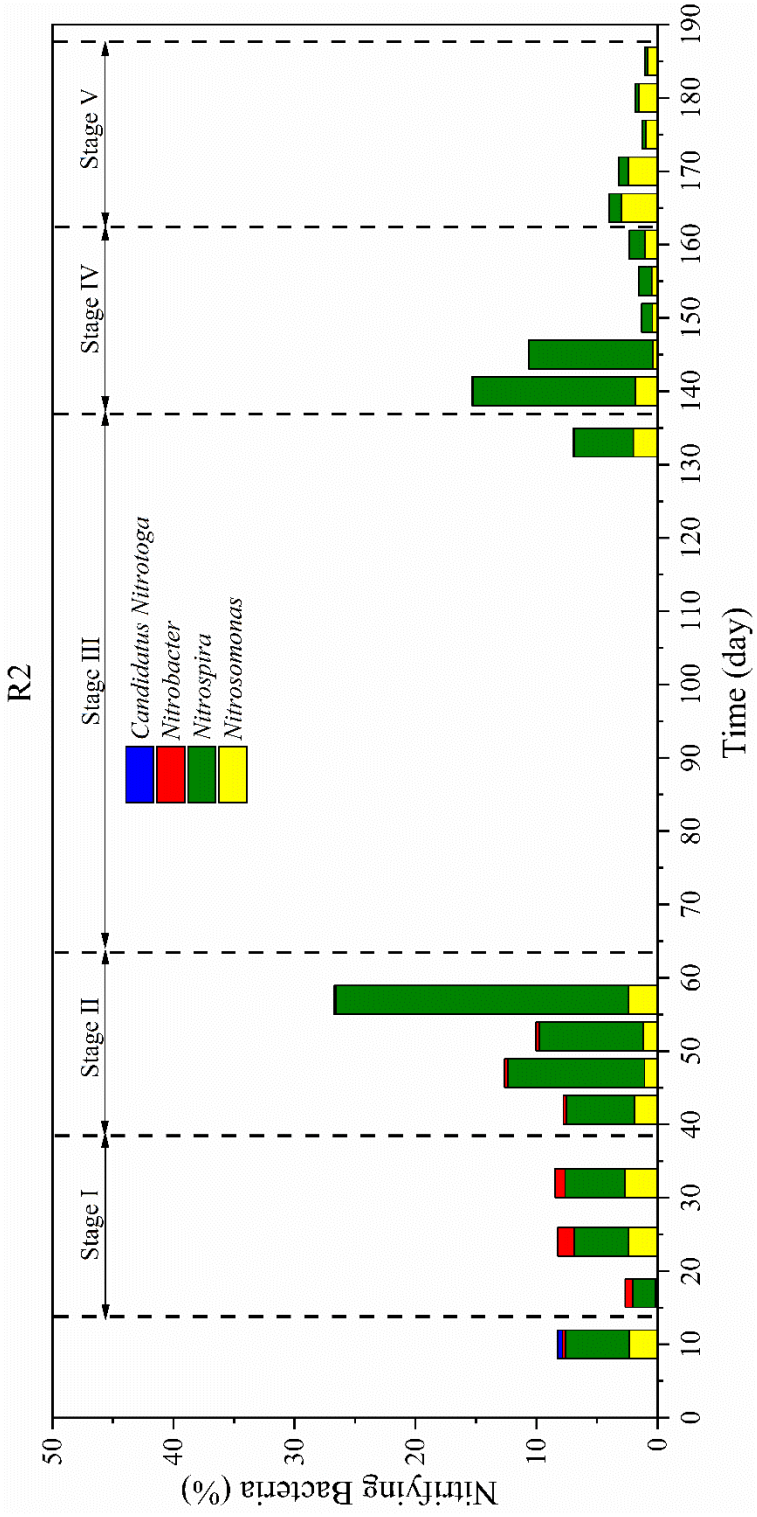

Figure 5-8 Percentage changes of nitrifying bacteria in sludge samples taken from R2. Stage I: R2 (4 mg-LVX/L); Stage IV: R2 (128 mg-LVX/L).

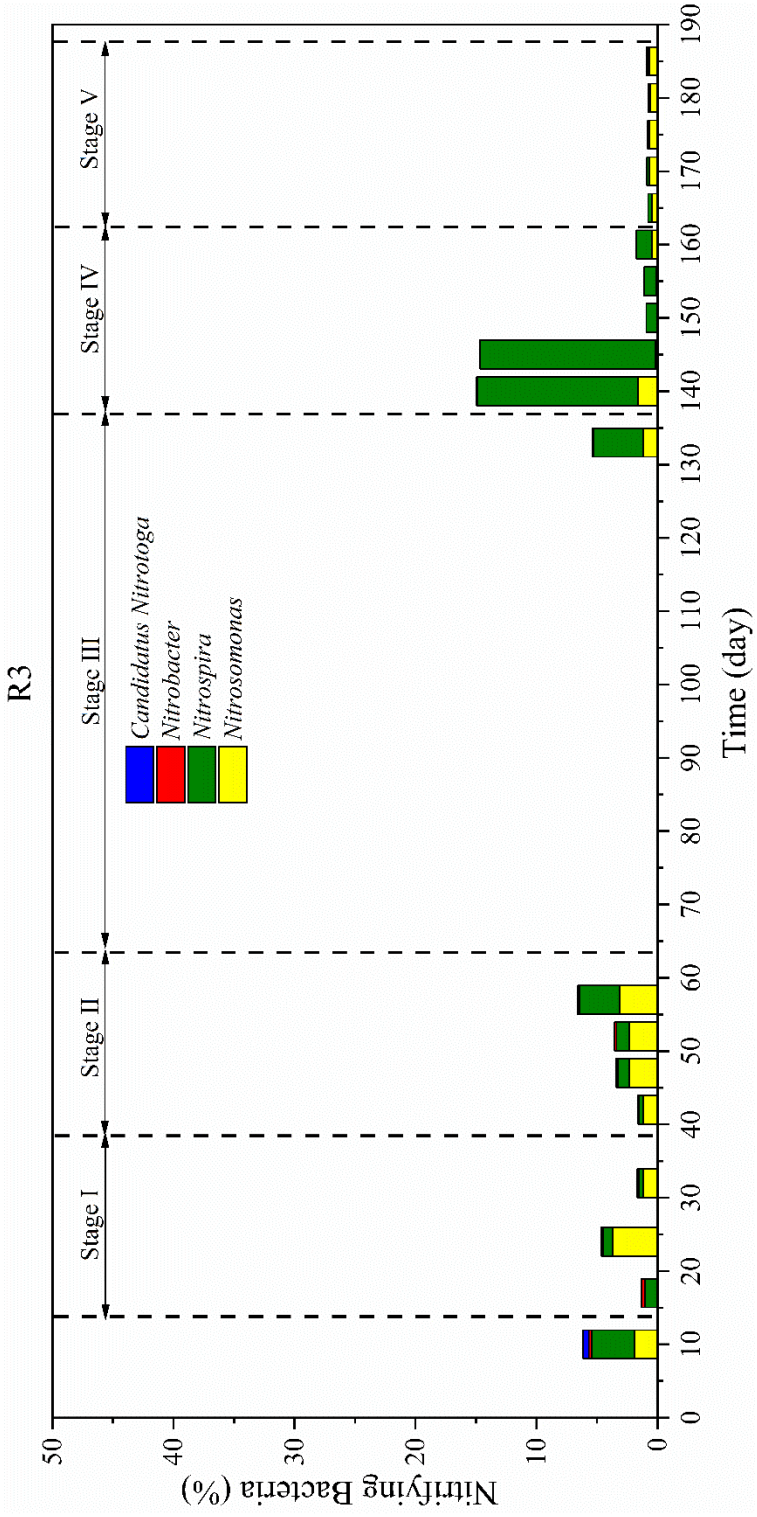

Figure 5-9 Percentage changes of nitrifying bacteria in sludge samples taken from R3. Stage I: R3 (16 mg-LVX/L); Stage IV: R3 (128 mg-LVX/L).

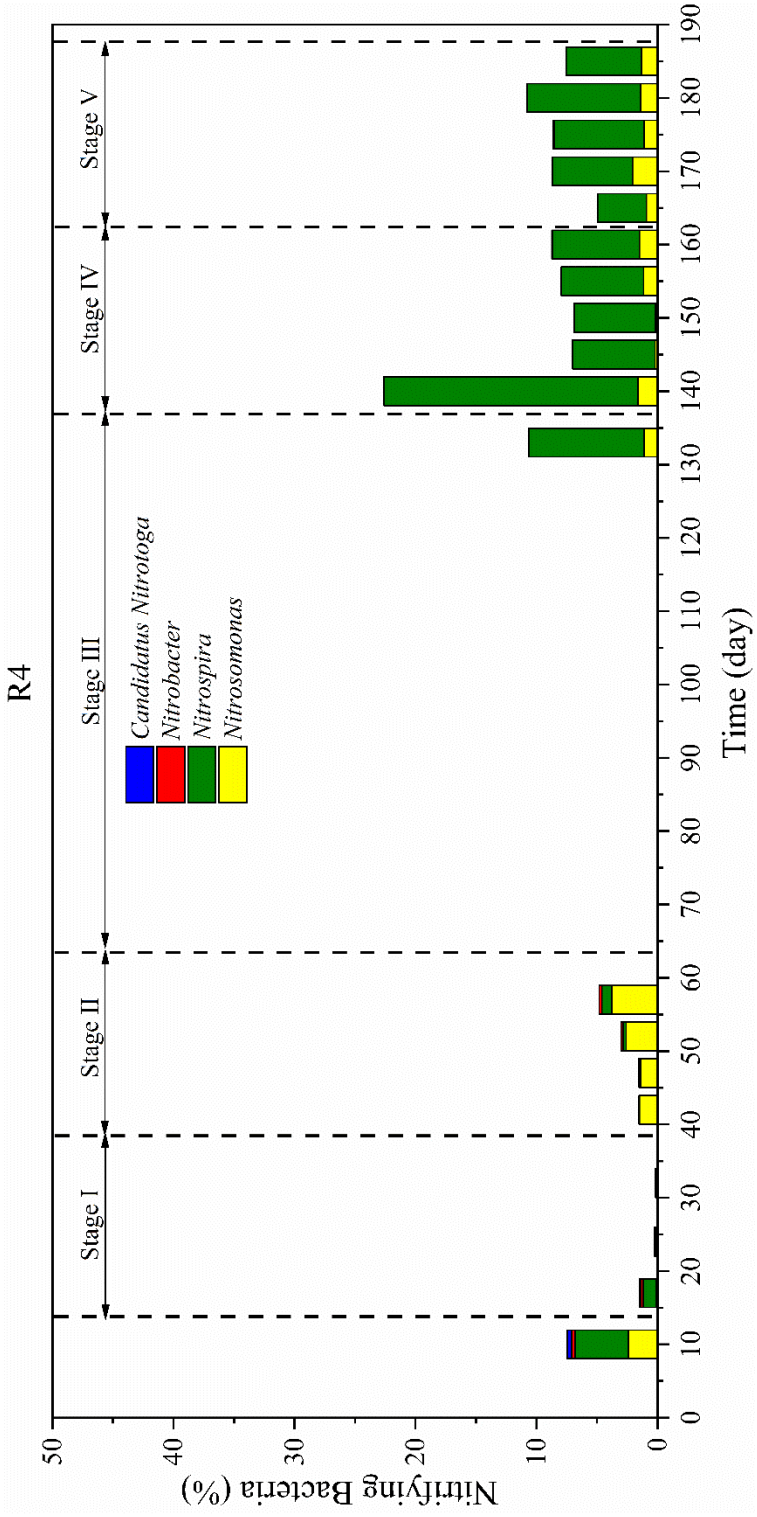

Figure 5-10 Percentage changes of nitrifying bacteria in sludge samples taken from R4. Stage I: R4 (128 mg-LVX/L); Stage IV: R4 (4 mg-LVX/L).

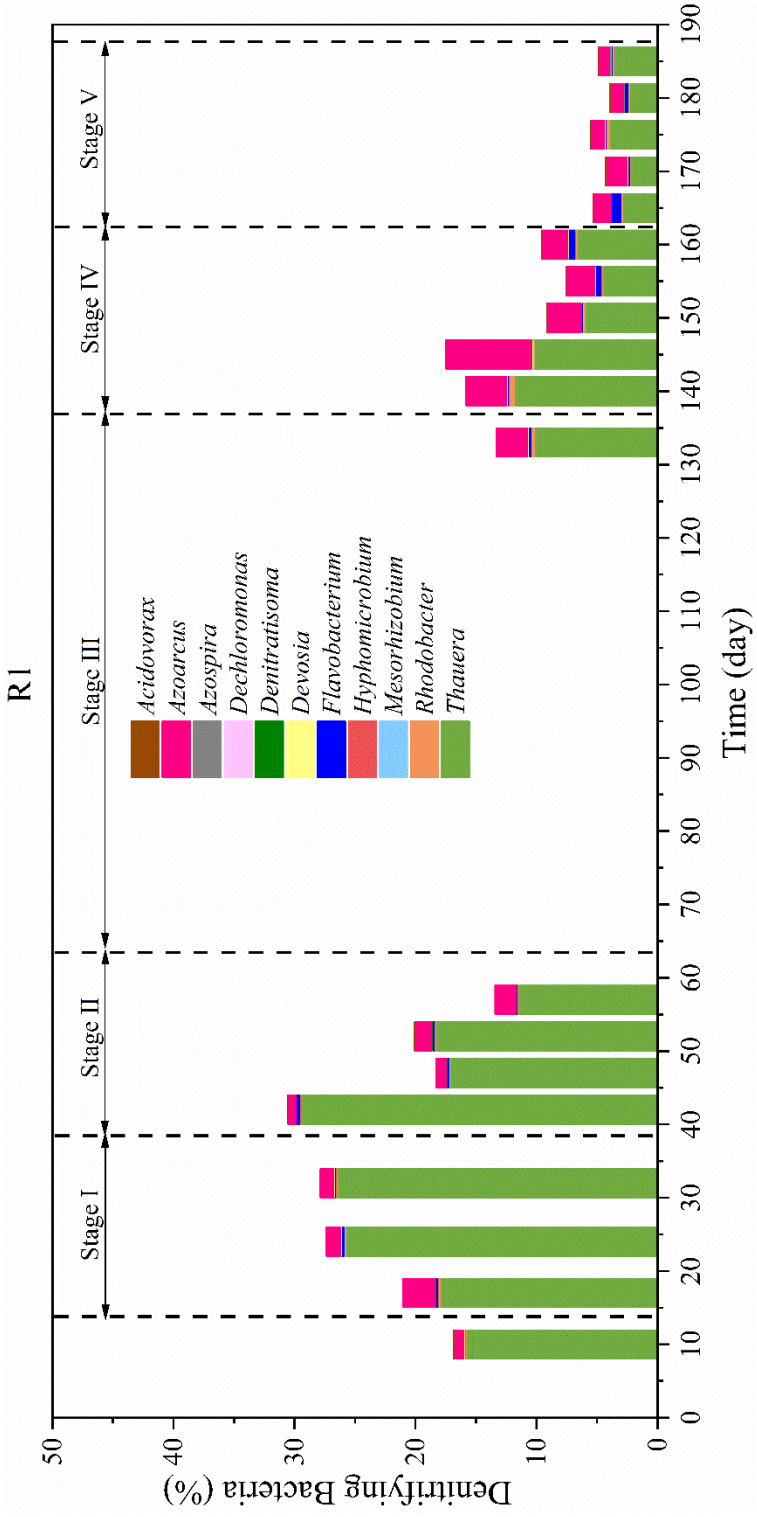

Figure 5-11 Percentage changes of denitrifying bacteria in sludge samples taken from R1. Stage I: R1 (0 mg-LVX/L); Stage IV: R1 (0 mg-LVX/L).

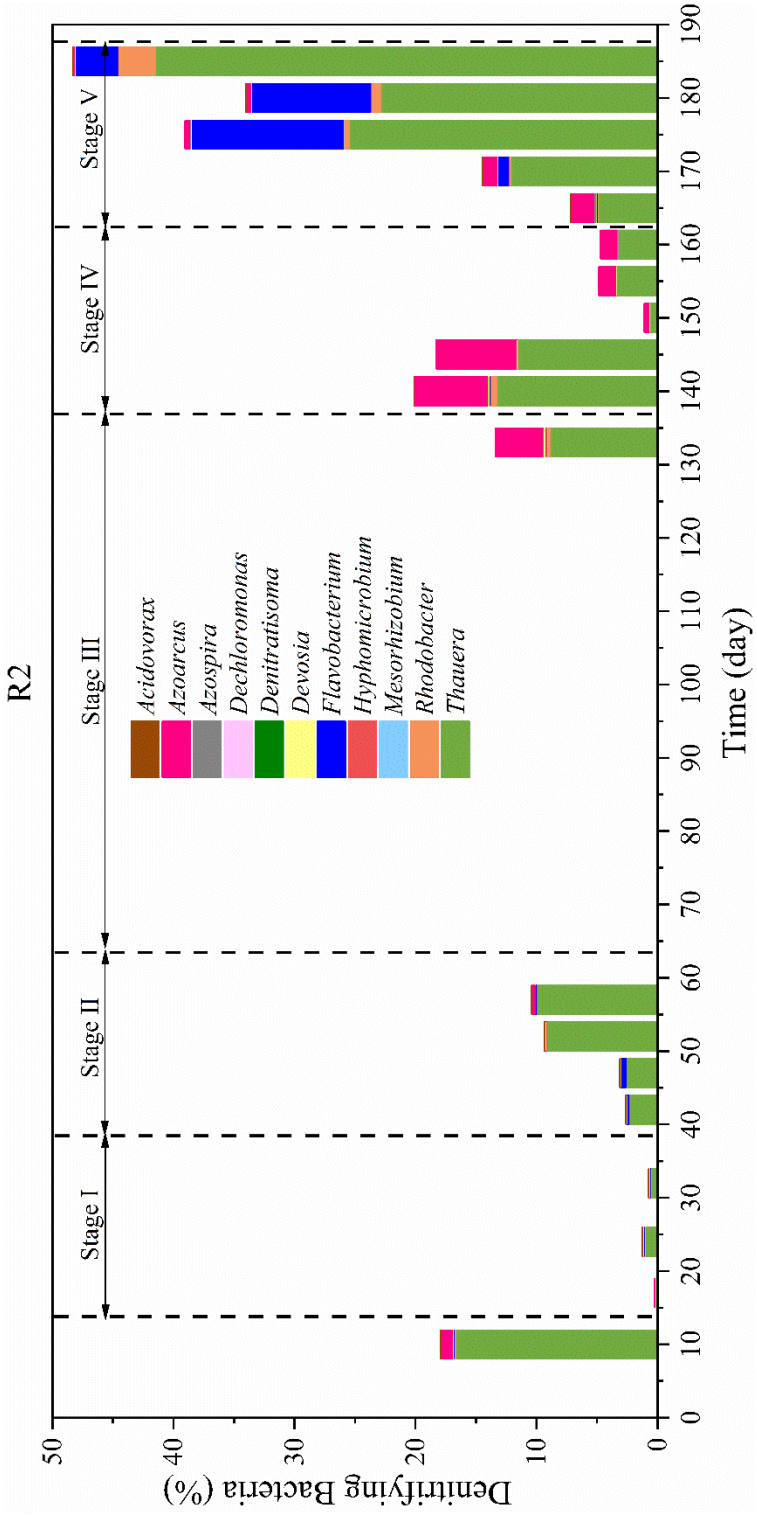

Figure 5-12 Percentage changes of denitrifying bacteria in sludge samples taken from R2. Stage I: R2 (4 mg-LVX/L); Stage IV: R2 (128 mg-LVX/L).

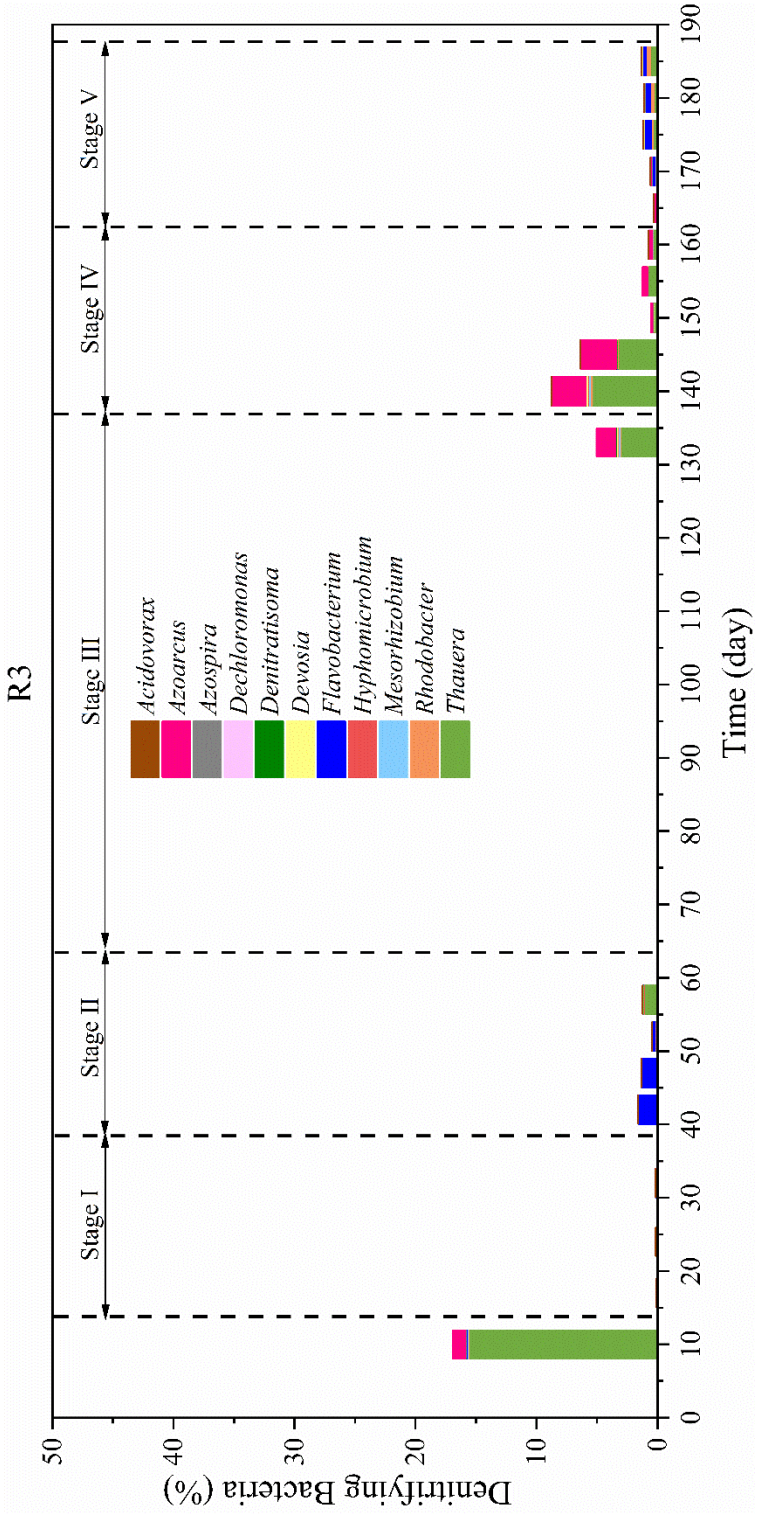

Figure 5-13 Percentage changes of denitrifying bacteria in sludge samples taken from R3. Stage I: R3 (16 mg-LVX/L); Stage IV: R3 (128 mg-LVX/L).

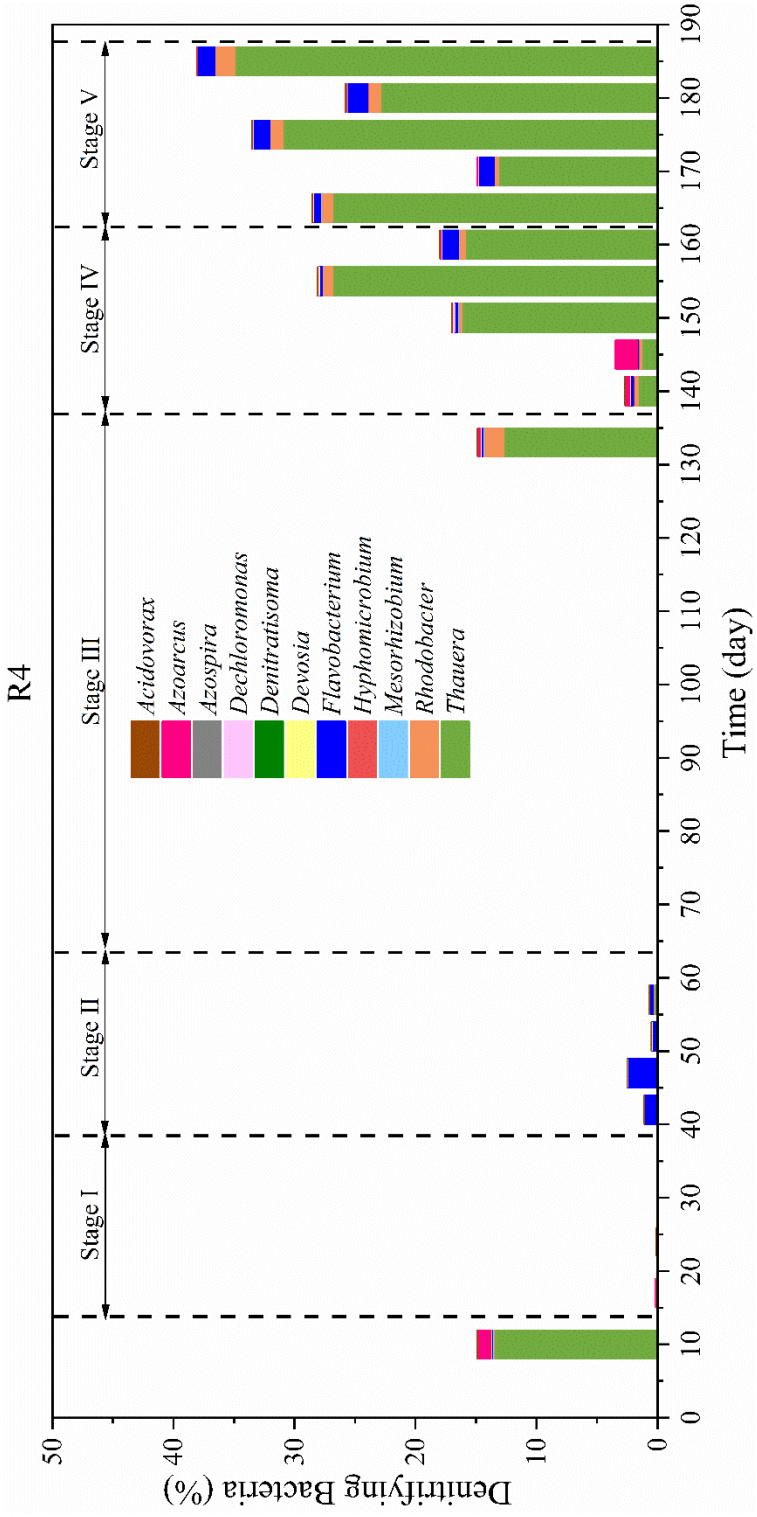

Figure 5-14 Percentage changes of denitrifying bacteria in sludge samples taken from R4. Stage I: R4 (128 mg-LVX/L); Stage IV: R4 (4 mg-LVX/L).

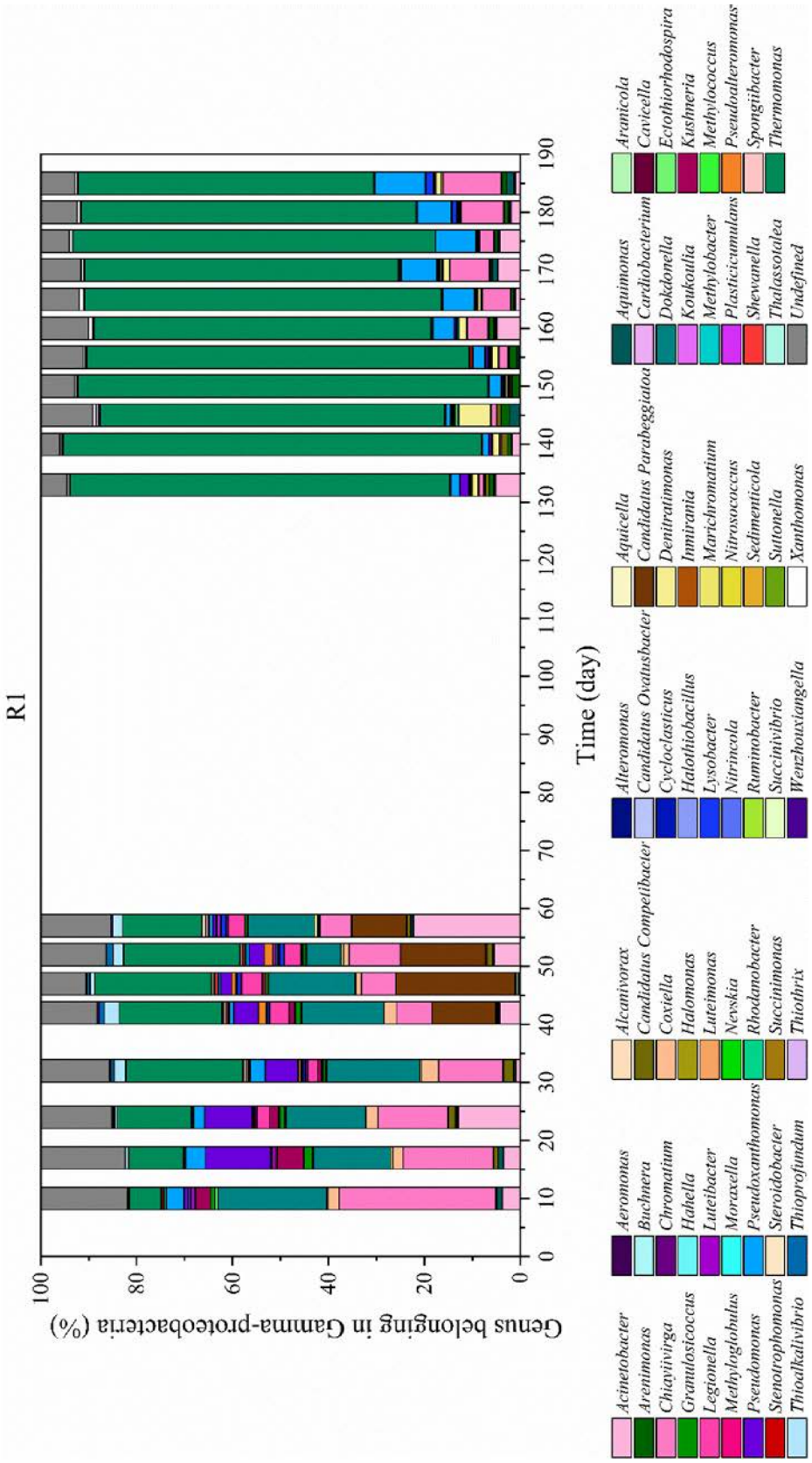

Figure 5-15 Genus belonging in Gamma-proteobacteria of the samples taken from R1.

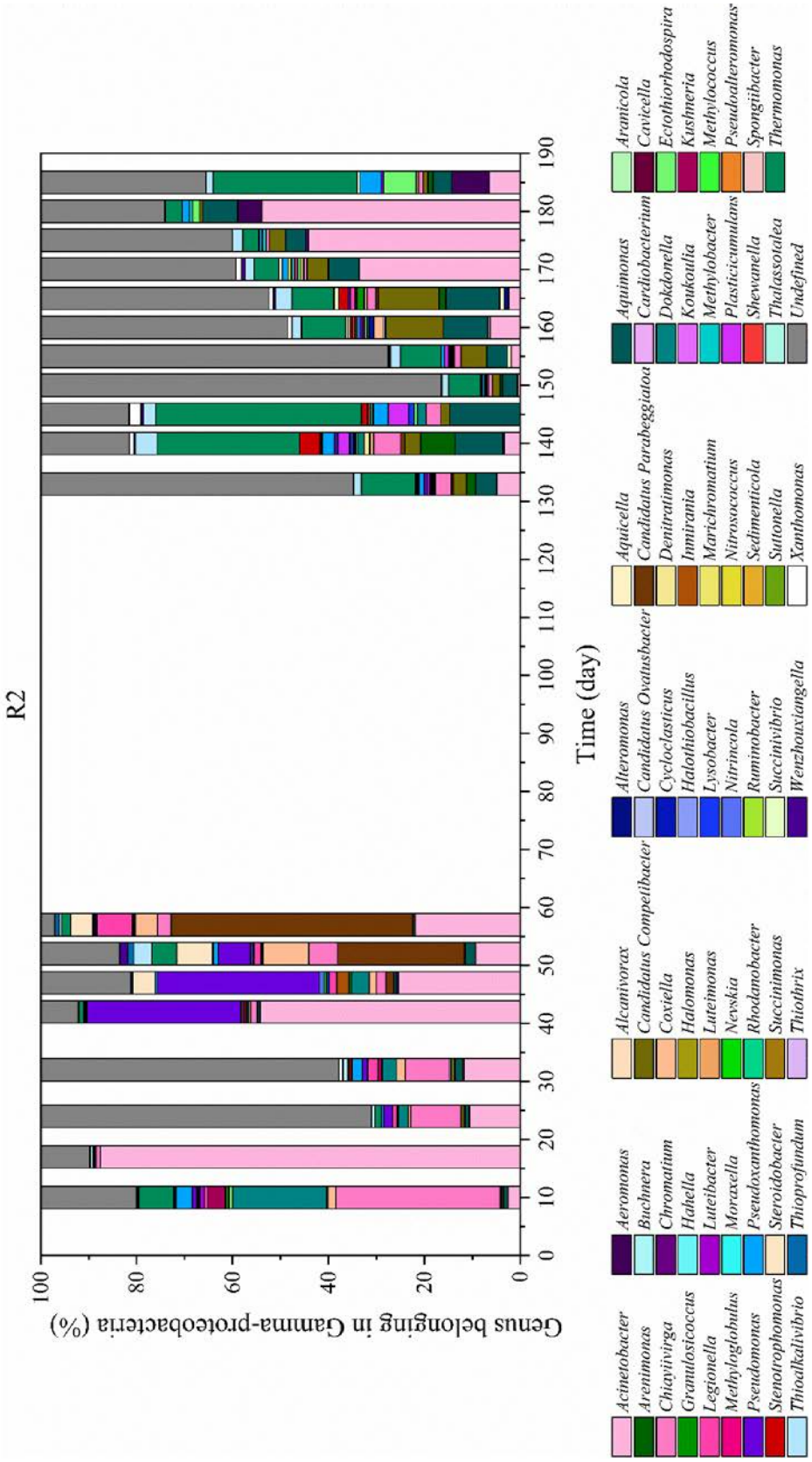

Figure 5-16 Genus belonging in Gamma-proteobacteria of the samples taken from R2.

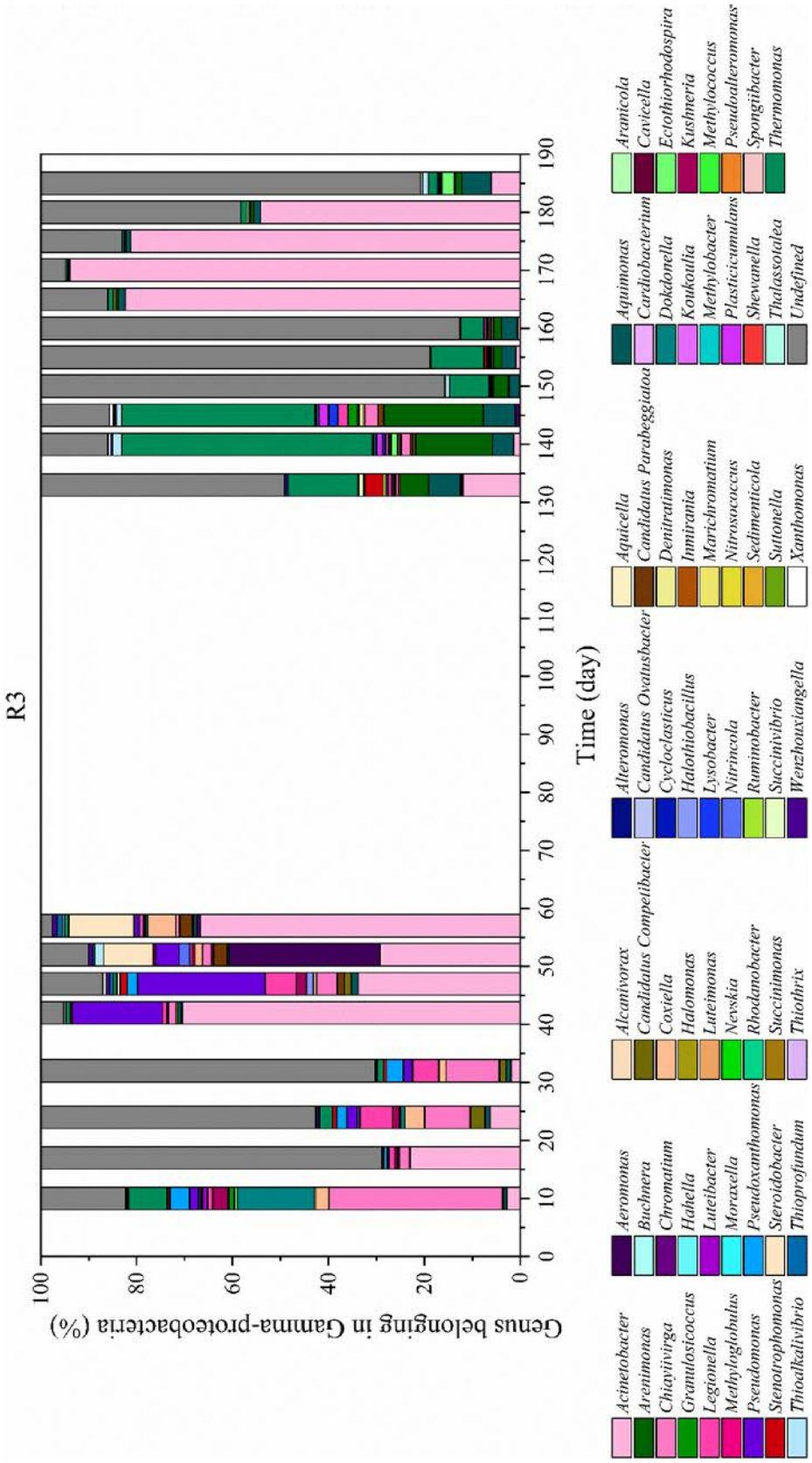

Figure 5-17 Genus belonging in Gamma-proteobacteria of the samples taken from R3.

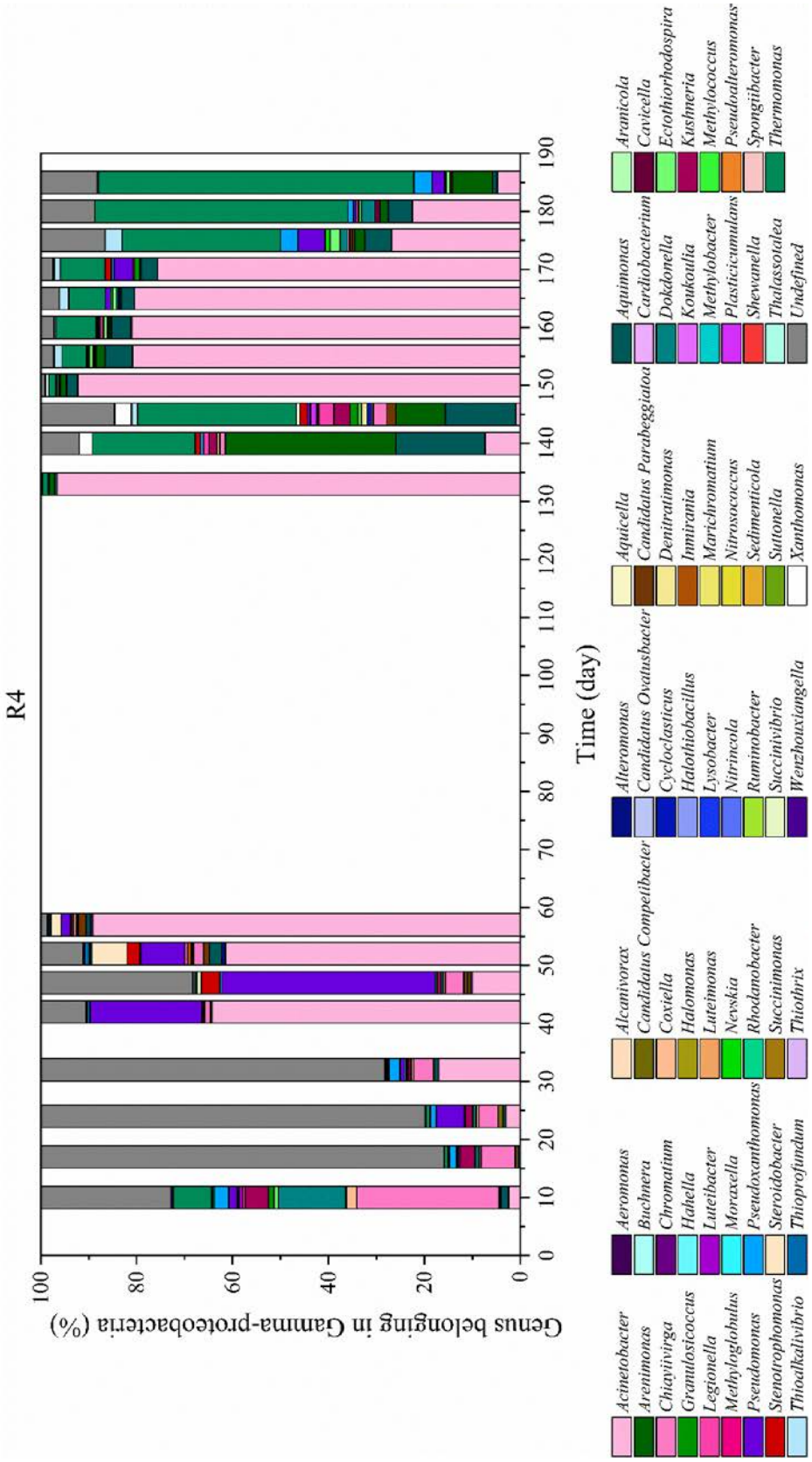

Figure 5-18 Genus belonging in Gamma-proteobacteria of the samples taken from R4.

Chapter 6 Conclusions and Future Research

6.1. Conclusions

In the Chapter 1, according to lots of reported researches, antibiotics and their emergence in the environment have been revealed. Also, antibiotic resistance with its mechanisms, the antibiotic residues situation in WWTPs and the effect of treatment processes on antibiotics' removal have been much more clearly. Many researches have been focused on antibiotics contamination, but most of the antibiotics are made by microorganisms. That is why the antibiotics from human activity are hard to analyze. So, we focus on quinolones that is completely synthetic antibiotics. No degrader and producer of quinolones live in the environment as so far. To mimic the uncontrolled or accidental discharge of LVX to the WWTPs, both exposure and re-exposure of varying LVX concentrations were considered and designed in the study. In addition to the effect on nutrients removal from the wastewater, dynamic changes of microbial communities during both exposure and re-exposure of LVX were also recorded and analyzed. This study aimed to shed light on the acclimation mechanisms of bacteria to acute and chronic/fluctuated LVX levels in SBRs.

In the Chapter 2, the resistance pattern test (MIC and MBC) of the sludge samples taken from the four SBRs was performed before LVX exposure operation. The results of minimum inhibitory concentration (MIC, 32 mg-LVX/L) and minimum bactericidal concentration (MBC, 512 mg-LVX/L) of the sampled sludge showed that the LVX resistance/tolerance for bacterial growth has already existed in sludge from the actual WWTPs (The LVX concentration levels of the directly filtered inoculated sludge samples were in the range of 0.09 to 0.76 mg-LVX/L in this study.). The result of this chapter is also useful for exposure/re-exposure operation concentrations' deciding.

In the Chapter 3 and Chapter 4, better understanding of the effects of acute and chronic/fluctuated LVX on the wastewater treatment performance in SBRs was focused in this study. LVX addition could inhibit the uptake of organics by the microorganisms, and a better dissolved organic carbon (DOC) removal performance during re-exposure was noticed in the reactor with lower LVX exposure concentration experience. Still, at least 50% of the influent LVX remained in the effluent. NH_4-N removals were remarkably affected, most probably the inhibition of ammonia-oxidizing bacteria (AOB) by the high concentration of LVX, resulted in much lower nitritation efficiency. Ammonia oxidation ability recovered much slower during re-exposure. Overall, the NO_2-N accumulation as well as lower NO_3-N production were

observed during the exposure/re-exposure to the higher concentration of LVX. During Stage V, NO_2-N concentration in both R2 and R3 could not recover as the beginning level and the nitratation processes are still inhibited in R2 and R3. TN increased with the LVX exposure/re-exposure, might not only be attributable to inhibited denitrification, but also the addition of LVX ($C_{18}H_{20}N_3O_4F$) which contains 11.7% of N (w/w). Re-exposure to 128 mg-LVX/L exerts more negative effects on the TN level of R3 than R2.

In the Chapter 5, in addition to the changes in the performance of wastewater treatment, the dynamic changes of microbial communities were also quantified to further investigate the adaptation mechanisms of bacteria community. Involved bacteria to nitrogen removal were affected by LVX exposure leading to deterioration of nitrogen removal function in treatment, but after the exposure stopped, the bacteria recovered to restart the nitrogen removal. The recovery of biological nutrients removal performance was retarded by LVX exposure, due to the fact that the key bacteria, i.e. *Nitrosomonas* sp., (AOB) and *Nitrospira* sp., nitrite-oxidizing bacteria (NOB) decreased, and that *Thauera* sp., the predominant denitrifiers were reduced during the LVX exposure period. However, after stopping exposure these population was quickly increased and thus the performance was recovered. The results of the non-metric multidimensional scaling (NMDS) and microbial community by sequencing showed the LVX concentration was a crucial factor to phase change of bacterial community controlling the quantitative evolution of the communities in the reactor systems. This effect was more pronounced as the LVX concentration was higher. Proteobacteria phylum always dominated the control reactor, but the proportion shifted dramatically with the addition of LVX. *Acinetobacter* sp. that important bacteria for multi-drug resistant on chemotherapy increased the abundance during high concentration of LVX exposure, but during no exposure time, that decreased due to being beaten in a competence. The results from this study suggest the control of residual antibiotics concentration in WWTPs is important to control antibiotic resistant bacteria (ARB) in WWTPs.

This study illustrated the importance of the control of residual antibiotics concentration not to be ARB sources and showed the resilience on residual antibiotics exposure by the adaptation of bacterial community to residual antibiotics contamination for nitrogen removal. The findings from this work are expected to provide good guidance on performance recovery of WWTPs when encountering accidental loading of antibiotics like LVX. Also, the results from this work could shed light on better understanding the acclimation mechanisms of bacteria and bacterial community to fluctuated LVX levels exposure situation during wastewater

treatment.

6.2. Future Research

(1) In the further experiments, the bacteria of the sludge using the plates (with/without LVX) could be isolated before, during and after the exposure/re-exposure.

(2) The appearance rate of multi-drug resistance (chloramphenicol, erythromycin, tetracycline) could be clarified and the changes of isolated bacteria in multi-drug resistance ability before, during and after exposure/re-exposure could be also confirmed.

(3) Further studies are also needed to clarify the ARB dynamics in real wastewater treatment plant and effluent after disinfection by chloride from the wastewater treatment plant and promotion of multi-drug resistant, especially efflux pump by antibiotic exposure.

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